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Active and inactive CC-chemokine receptors and nucleic acid molecules encoding said receptor

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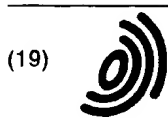
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(54) **Active and inactive CC-chemokine receptors and nucleic acid molecules encoding said
receptor**

(57) The present invention is related to new pep-
tides and the nucleic acid molecules encoding said pep-
tides. The present invention concerns also the vector
comprising said nucleic acid molecules, cells trans-
formed by said vector, inhibitors directed against said
peptides or said nucleic acid molecules, a pharmaceu-

tical composition and a diagnostic and/or dosage device
comprising said products, and non human transgenic
animals expressing the peptides according to the inven-
tion or the nucleic acid molecules encoding said pep-
tides.

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Description**Field of the present invention.**

- 5 [0001] The present invention concerns new peptides and the nucleic acid molecules encoding said peptides, the vector comprising said nucleic acid molecules, the cells transformed by said vector, inhibitors directed against said peptides or said nucleic acid molecules, a pharmaceutical composition and a diagnostic and/or dosage device comprising said products, and non human transgenic animals expressing the peptides according to the invention or the nucleic acid molecules encoding said peptides.
- 10 [0002] The invention further provides a method for determining ligand binding, detecting expression, screening for drugs binding specifically to said peptides and treatments involving the peptides or the nucleic acid molecules according to the invention.

Technological background and state of the art.

- 15 [0003] Chemotactic cytokines, or chemokines, are small signalling proteins that can be divided in two subfamilies (CC- and CXC-chemokines) depending on the relative position of the first two conserved cysteines. Interleukin 8 (IL-8) is the most studied of these proteins, but a large number of chemokines (Regulated on Activation Normal T-cell Expressed and Secreted (RANTES), Monocyte Chemoattractant Protein 1 (MCP-1), Monocyte Chemoattractant Protein 2 (MCP-2), Monocyte Chemoattractant Protein 3 (MCP-3), Growth-Related gene product α (GRO α), Growth-Related gene product β (GRO β), Growth-Related gene product γ (GRO γ), Macrophage Inflammatory Protein 1 α (MIP-1 α) and β , etc.) has now been described [4]. Chemokines play fundamental roles in the physiology of acute and chronic inflammatory processes as well as in the pathological dysregulations of these processes, by attracting and stimulating specific subsets of leucocytes [32]. RANTES for example is a chemoattractant for monocytes, memory T-cells and eosinophils, and induces the release of histamine by basophils. MCP-1, released by smooth muscle cells in arterio-sclerotic lesions, is considered as the factor (or one of the factors) responsible for macrophage attraction and, therefore, for the progressive aggravation of the lesions [4].
- 20 [0004] MIP-1 α , MIP-1 β and RANTES chemokines have recently been described as major HIV-suppressive factors produced by CD8⁺ T-cells [9]. CC-chemokines are also involved in the regulation of human myeloid progenitor cell proliferation [6, 7].
- 25 [0005] Recent studies have demonstrated that the actions of CC- and CXC-chemokines are mediated by subfamilies of G protein-coupled receptors. To date, despite the numerous functions attributed to chemokines and the increasing number of biologically active ligands, only six functional receptors have been identified in human. Two receptors for interleukin-8 (IL-8) have been described [20, 29]. One (IL-8RA) binds IL-8 specifically, while the other (IL-8RB) binds IL-8 and other CXC-chemokines, like GRO. Among receptors binding CC-chemokines, a receptor, designated CC-chemokine receptor 1 (CCR1), binds both RANTES and MIP-1 α [31], and the CC-chemokine receptor 2 (CCR2) binds MCP-1 and MCP-3 [8, 44, 15]. Two additional CC-chemokine receptors were cloned recently : the CC-chemokine receptor 3 (CCR3) was found to be activated by RANTES, MIP-1 α and MIP-1 β [10]; the CC-chemokine receptor 4 (CCR4) responds to MIP-1, RANTES and MCP-1 [37]. In addition to these six functional receptors, a number of orphan receptors have been cloned from human and other species, that are structurally related to either CC- or CXC-chemokine receptors. These include the human BLR1 [13], EBI1 [5], LCR1 [21], the mouse MIP-1 RL1 and MIP-1 RL2 [17] and the bovine PPR1 [25]. Their respective ligand(s) and function(s) are unknown at present.
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Summary of the invention.

- 45 [0006] The present invention relates to a peptide having at least an amino acid sequence which presents more than 80% homology with the amino acid sequence as represented in SEQ ID NO. 1 shown in figure 1.
- [0007] The present invention also relates to a peptide as mentioned above, having at least an amino acid sequence which presents more than 90% homology with the amino acid sequence as represented in SEQ ID NO. 1 shown in figure 1.
- 50 [0008] The present invention further relates to the peptide as mentioned above, having the amino acid sequence of SEQ ID NO. 1 or a portion thereof.
- [0009] The present invention also relates to the peptide as mentioned above, having at least an amino acid sequence which presents more than 80% homology with the amino acid sequence as represented in SEQ ID NO. 2 shown in figure 1.
- 55 [0010] The present invention further relates to a peptide as mentioned above, having at least an amino acid sequence which presents more than 90% homology with the amino acid sequence as represented in SEQ ID NO. 2 shown in figure 1.

[0011] The present invention also relates to a peptide as mentioned above, having the amino acid sequence of SEQ ID NO. 2 or a portion thereof.

[0012] The present invention further relates to a peptide as mentioned above, having at least an amino acid sequence which presents more than 80% homology with the amino acid sequence as represented in SEQ ID NO. 3 shown in figure 1.

[0013] Further, the present invention also relates to a peptide as mentioned above, having at least an amino acid sequence which presents more than 90% homology with the amino acid sequence as represented in SEQ ID NO. 3 shown in figure 1.

[0014] The present invention also relates to a peptide as mentioned above, having the amino acid sequence of SEQ ID NO. 3 or a portion thereof.

[0015] The present invention further relates to a peptide as mentioned above, characterised in that it is a CC chemokine receptor.

[0016] The present invention also relates to a peptide as mentioned above, characterised in that the CC chemokine receptor is stimulated by the MIP-1 β chemokine at a concentration less or equal to 10 nM.

[0017] The present invention further relates to a peptide as mentioned above, characterised in that the CC chemokine receptor is stimulated by the MIP-1 α or RANTES chemokines.

[0018] The present invention further relates to a peptide as mentioned above, characterised in that the CC chemokine receptor is not stimulated by the MCP-1, MCP-2, MCP-3, IL-8 and GRO α chemokines.

[0019] The present invention further relates to a peptide as mentioned above, characterised in that it is a receptor of HIV-1 and/or HIV-2 viruses or a portion of said HIV viruses.

[0020] The present invention also relates to a peptide as mentioned above, characterised in that it is an inactive CC chemokine receptor.

[0021] The present invention also relates to a peptide as mentioned above, characterised in that it is an inactive receptor, which is not a receptor of HIV-1 and/or HIV-2 viruses or a portion of said HIV viruses.

[0022] The present invention also relates to a peptide as mentioned above, being a human receptor.

[0023] The present invention also relates to a nucleic acid molecule having more than 80%, preferably more than 90%, homology with one of the nucleic acid sequences SEQ ID NO. 1, SEQ ID NO. 2 and SEQ ID NO. 3 shown in figure 1.

[0024] The present invention further relates to a nucleic acid as mentioned above, which has at least the nucleic acid sequence SEQ ID NO. 1, SEQ ID NO. 2 or SEQ ID NO. 3 shown in figure 1 or a portion (such as a probe or a primer) thereof.

[0025] The present invention further relates to a nucleic acid encoding a peptide as mentioned above.

[0026] The present invention also relates to a nucleic acid molecule as mentioned above, which is a cDNA molecule or a genomic DNA molecule.

[0027] The present invention also relates to a vector comprising the nucleic acid molecule as mentioned above.

[0028] The present invention further relates to a vector as mentioned above, adapted for expression in a cell, which comprises the regulatory elements necessary for expression of the nucleic acid molecule in said cell operatively linked to the nucleic acid molecule as mentioned above as to permit expression thereof.

[0029] The present invention also relates to a vector as mentioned above, wherein the cell is chosen among the group consisting of bacterial cells, yeast cells, insect cells or mammalian cells.

[0030] The present invention further relates to a vector as mentioned above, wherein the vector is a plasmid or a virus.

[0031] The present invention also relates to the above-mentioned vector being a virus, chosen among the group consisting of baculoviruses, adenoviruses or semliki forest viruses.

[0032] The present invention further relates to a vector as mentioned above, being the pcDNA3 plasmid.

[0033] The present invention also relates to a cell, preferably a human cell, comprising the above-mentioned vector.

[0034] The present invention further relates to the above-mentioned cell, characterised in that it is transformed also by another vector encoding a protein enhancing the functional response in said cell, preferably said protein being the G α 15 or the G α 16 protein.

[0035] The present invention also relates to a cell as mentioned above, wherein the cell is a mammalian cell, such as a non neuronal cell in origin, which is preferably chosen among the group consisting of CHO-K1, HEK293, BHK21, COS-7 cells.

[0036] The present invention further relates to a cell as mentioned above, being the cell CHO-K1-pEFIN hCCR5-1/16.

[0037] The present invention further relates to a nucleic acid probe comprising a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridising with a unique sequence included within the nucleic acid molecule as mentioned above.

[0038] The present invention further relates to an antisense oligonucleotide having a sequence capable of specifically hybridising to a nucleic acid molecule as mentioned above, so as to prevent translation of said nucleic acid molecule.

[0039] The present invention also relates to an antisense oligonucleotide having a sequence capable of specifically

hybridising to the DNA molecule as mentioned above or a portion thereof.

[0040] The present invention further relates to an antisense oligonucleotide as mentioned above, comprising chemical analogs of nucleotides.

[0041] The present invention also relates to a ligand capable of binding to the peptide as mentioned above with the proviso that it is not a known "natural ligand" of said peptide, which is preferably chosen among the group consisting of MIP-1 β , MIP-1 α or RANTES CC-chemokines, HIV viruses or a portion of said HIV viruses.

[0042] The present invention also relates to an anti-ligand capable of competitively inhibiting the binding of the known "natural ligand" to the peptide as mentioned above.

[0043] The present invention further relates to a ligand as mentioned above, which is an antibody.

[0044] The present invention also relates to a ligand as mentioned above, which is an antibody.

[0045] The present invention further relates to an antibody as mentioned above, which is a monoclonal antibody.

[0046] The present invention also relates to a monoclonal antibody as mentioned above, directed to an epitope of the peptide as mentioned above, present on the surface of a cell expressing said peptide.

[0047] The present invention also relates to a cell producing the monoclonal antibody as mentioned above being the cell AchCCR5-SAB1A7.

[0048] The present invention further relates to a pharmaceutical composition comprising an amount of the above-mentioned oligonucleotide, effective to decrease activity of the peptide as mentioned above by passing through a cell membrane and binding specifically with mRNA encoding said peptide in the cell so as to prevent its translation, and a pharmaceutically acceptable carrier capable of passing through a cell membrane.

[0049] The present invention further relates to the above-mentioned pharmaceutical composition, wherein the oligonucleotide is coupled to a substance which inactivates mRNA.

[0050] The present invention also relates to the above-mentioned pharmaceutical composition, wherein the substance which inactivates mRNA is a ribozyme.

[0051] Further, the present invention relates to the above-mentioned pharmaceutical composition, wherein the pharmaceutically acceptable carrier comprises a structure which binds to a receptor on a cell capable of being taken up by cell after binding to the structure.

[0052] The present invention further relates to a pharmaceutical composition which comprises an effective amount of the anti-ligand as mentioned above, effective to block binding of a ligand to the above-mentioned peptide and a pharmaceutically acceptable carrier.

[0053] The present invention further relates to a transgenic non human mammal expressing the nucleic acid molecule as mentioned above.

[0054] The present invention also relates to a transgenic non human mammal comprising an homologous recombination knockout of the native peptide as mentioned above.

[0055] Further, the invention relates to a transgenic non human mammal whose genome comprises anti sense nucleic acid complementary to the nucleic acid molecule as mentioned above so placed as to be transcribed into antisense mRNA which is complementary to the nucleic acid molecule as mentioned above and which hybridises to said nucleic acid molecule thereby reducing its translation.

[0056] The invention also relates to a transgenic non human mammal as mentioned above, wherein the above-mentioned nucleic acid additionally comprises an inducible promoter.

[0057] Further, the invention relates to a transgenic non human mammal as mentioned above, wherein the above-mentioned nucleic acid additionally comprises tissue specific regulatory elements.

[0058] The present invention also relates to a transgenic non human mammal as mentioned above, which is a mouse.

[0059] The present invention also relates to a method for determining whether a ligand can specifically bind to a peptide as mentioned above, which comprises contacting a cell transfected with a vector expressing the nucleic acid molecule encoding said peptide with the ligand under conditions permitting binding of the ligand to such peptide and detecting the presence of any such ligand bound specifically to said peptide, thereby determining whether the ligand binds specifically to said peptide.

[0060] The present invention also relates to a method for determining whether a ligand can specifically bind to the peptide as mentioned above, which comprises preparing a cell extract from cells transfected with a vector expressing the nucleic acid molecule encoding said peptide, isolating a membrane fraction from the cell extract, contacting the ligand with the membrane fraction under conditions permitting binding of the ligand to such peptide and detecting the presence of any ligand bound to said peptide, thereby determining whether the compound is capable of specifically binding to said peptide.

[0061] The present invention further relates to a method for determining whether a ligand is an agonist of the peptide as mentioned above, which comprises contacting a cell transfected with a vector expressing the nucleic acid molecule encoding said peptide with the ligand under conditions permitting the activation of a functional receptor response from the cell and detecting by means of a bio-assay, such as a second messenger response, an increase in the peptide activity, thereby determining whether the ligand is a peptide agonist.

[0062] The present invention also relates to a method for determining whether a ligand is an agonist of the peptide as mentioned above, which comprises preparing a cell extract from cells transfected with a vector expressing the nucleic acid molecule encoding said peptide, isolating a membrane fraction from the cell extract, contacting the membrane fraction with the ligand under conditions permitting the activation of a functional peptide response and detecting by means of a bio-assay, such as a second messenger response, an increase in the peptide activity, thereby determining whether the ligand is a peptide agonist.

[0063] The present invention also relates to a method for determining whether a ligand is an antagonist of the peptide as mentioned above, which comprises contacting a cell transfected with a vector expressing the nucleic acid molecule encoding said peptide with the ligand in the presence of a known peptide agonist, under conditions permitting the activation of a functional peptide response and detecting by means of a bio-assay, such as a second messenger response, a decrease in the peptide activity, thereby determining whether the ligand is a peptide antagonist.

[0064] The present invention further relates to a method for determining whether a ligand is an antagonist of the peptide as mentioned above, which comprises preparing a cell extract from cells transfected with a vector expressing the nucleic acid molecule encoding said peptide, isolating a membrane fraction from the cell extract, contacting the membrane fraction with the ligand in the presence of a known peptide agonist, under conditions permitting the activation of a functional peptide response and detecting by means of a bio-assay, such as a second messenger response, a decrease in the peptide activity, thereby determining whether the ligand is a receptor antagonist.

[0065] The present invention also relates to a method as mentioned above, wherein the second messenger assay comprises measurement of calcium ions (Ca^{2+}), inositol phosphates (such as IP_3), diacylglycerol (DAG) or cAMP.

[0066] The present invention also relates to a method as mentioned above, wherein the cell is a mammalian cell, preferably non neuronal in origin, and chosen among the group consisting of CHO-K1, HEK293, BHK21 and COS-7 cells.

[0067] The present invention further relates to a method as mentioned above, wherein the ligand is not previously known.

[0068] Further, the present invention relates to a ligand detected by the above-mentioned method.

[0069] The present invention also relates to a pharmaceutical composition which comprises the above-mentioned ligand and a pharmaceutically acceptable carrier.

[0070] The present invention relates to a method of screening drugs to identify drugs which specifically bind to the peptide as mentioned above on the surface of the cell, which comprises contacting a cell transfected with a vector expressing the nucleic acid molecule encoding said peptide with a plurality of drugs under conditions permitting binding of said drugs to the peptide, and determining those drugs which specifically bind to the transfected cell, thereby identifying drugs which specifically bind to the peptide.

[0071] The present invention further relates to a method of screening drugs to identify drugs which specifically bind to the peptide as mentioned above on the surface of the cell, which comprises preparing a cell extract from cells transfected with a vector expressing the nucleic acid molecule encoding said peptide, isolating a membrane fraction from the cell extract, contacting the membrane fraction with a plurality of drugs and determining those drugs which bind to the transfected cell, thereby identifying drugs which specifically bind to said peptide.

[0072] The present invention further relates to a method of screening drugs to identify drugs which act as agonists of the peptide as mentioned above, which comprises contacting a cell transfected with a vector expressing the nucleic acid molecule encoding said peptide with a plurality of drugs under conditions permitting the activation of a functional peptide response, and determining those drugs which activates such peptide using a bio-assay, such as a second messenger response, thereby identifying drugs which act as peptide agonists.

[0073] The present invention further relates to a method of screening drugs to identify drugs which act as agonists of the peptide as mentioned above, which comprises preparing a cell extract from cells transfected with a vector expressing the nucleic acid molecule encoding said peptide, isolating a membrane fraction from the cell extract, contacting the membrane fraction with a plurality of drugs under conditions permitting the activation of a functional peptide response, and determining those drugs which activate such peptide using a bio-assay, such as a second messenger response, thereby identifying drugs which act as peptide agonists.

[0074] Further, the present invention relates to a method of screening drugs to identify drugs which act as antagonists of the peptide as mentioned above, which comprises contacting a cell transfected with a vector expressing the nucleic acid molecule encoding said peptide with a plurality of drugs in the presence of a known peptide agonist, under conditions permitting the activation of a functional peptide response, and determining those drugs which inhibit the activation of the peptide using a bio-assay, such as a second messenger response, thereby identifying drugs which act as peptide antagonists.

[0075] The present invention also relates to a method of screening drugs to identify drugs which act as antagonists of the peptide as mentioned above, which comprises preparing a cell extract from cells transfected with a vector expressing the nucleic acid molecule encoding said peptide, isolating a membrane fraction from the cell extract, contacting the membrane fraction with a plurality of drugs in presence of a known peptide agonist, under conditions permitting

the activation of a functional peptide response, and determining those drugs which inhibit the activation of the peptide using a bio-assay, such as a second messenger response, thereby identifying drugs which act as peptide antagonists.

[0076] The present invention also relates to a method as mentioned above, wherein the functional response detected by means of a bio-assay is detected and measured by a microphysiometer.

5 [0077] Further, the present invention relates to a drug detected by any of the above-mentioned methods.

[0078] The present invention also relates to a pharmaceutical composition comprising the above-mentioned drug and a pharmaceutically acceptable carrier.

10 [0079] The present invention further relates to a method of detecting the expression of the peptide as mentioned above, by detecting the presence of mRNA coding for said receptor, which comprises obtaining total mRNA from the cell and contacting the mRNA so obtained with the nucleic acid probe as mentioned above under hybridising conditions, and detecting the presence of mRNA hybridised to the probe, thereby detecting the expression of the peptide by the cell.

[0080] The present invention also relates to a method of detecting the presence of the peptide as mentioned above on the surface of a cell, which comprises contacting the cell with the anti-ligand as mentioned above under conditions permitting binding of the antibody to the peptide, and detecting the presence of the antibody bound to the cell, thereby
15 detecting the presence of the peptide on the surface of the cell.

[0081] The present invention further relates to a method of determining the physiological effects of expressing varying levels of the peptide as mentioned above, which comprises producing a transgenic non human mammal as mentioned above whose levels of peptide expression are varied by use of an inducible promoter which regulates the peptide regulation.

20 [0082] Further, the present invention relates to a method of determining the physiological effects of expressing varying levels of the peptide as mentioned above, which comprises producing a panel of transgenic non human mammals as mentioned above, each expressing a different amount of said peptide.

[0083] The present invention further relates to a method for identifying an antagonist of the peptide as mentioned above capable of alleviating an abnormality in a subject wherein the abnormality is alleviated by decreasing the activity of the peptide, which comprises administering the antagonist to a transgenic non human mammal as mentioned above and determining whether the antagonist alleviates the physical and behavioural abnormalities displayed by the transgenic non human mammal as a result of peptide activity, thereby identifying the antagonist.

[0084] The present invention further relates to an antagonist identified by any of the above-mentioned methods.

25 [0085] The present invention also relates to a pharmaceutical composition comprising an antagonist as mentioned above and a pharmaceutically acceptable carrier.

[0086] The present invention further relates to a method for identifying an agonist of the peptide as mentioned above capable of alleviating an abnormality in a subject wherein the abnormality is alleviated by activation of said peptide, which comprises administering the agonist to a transgenic non human mammal as mentioned above and determining whether the antagonist alleviates the physical and behavioural abnormalities displayed by the transgenic non human mammal, the alleviation of the abnormalities indicating the identification of the agonist.

35 [0087] The present invention further relates to an agonist identified by the above-mentioned method.

[0088] The present invention also relates to a pharmaceutical composition comprising the above-mentioned agonist and a pharmaceutically acceptable carrier.

40 [0089] The present invention further relates to method for diagnosing a predisposition or a resistance to a disorder associated with the activity of a specific allele of the peptide as mentioned above, and/or associated with infectious agents, preferably the HIV-1 and/or HIV-2 viruses, present in a subject, which comprises :

a) obtaining nucleic acid molecules encoding said peptide from the cells of the subject,

45 b) possibly performing a restriction digest of said nucleic acid molecules with a panel of restriction enzymes,

c) possibly electrophoretically separating the resulting nucleic acid fragments on a sized gel,

50 d) contacting the resulting gel or the obtained nucleic acid molecules labelled with a nucleic acid probe with a detectable marker and capable of specifically hybridising to said nucleic acid molecule,

e) detecting labelled bands or in situ nucleic acid molecules which have hybridised to the said nucleic acid molecule labelled with a detectable marker to create a unique band pattern or in situ marking specific to the subject,

55 f) preparing other nucleic acid molecules encoding said peptide obtained from the cells of other subjects for diagnosis by step a-e, and

g) comparing the unique band pattern specific to the nucleic acid molecule of subjects suffering from the disorder

from step e and the nucleic acid molecule obtained for diagnosis from step f to determine whether the patterns are the same or different and to diagnose thereby predisposition or resistance to the disorder if the patterns are the same or different.

5 **[0090]** The present invention further relates to a method for diagnosing a predisposition or a resistance to a disorder associated with the activity of a specific allele of the peptide as mentioned above or the presence of said peptide at the surface of cells, and/or associated with infectious agents, preferably the HIV-1 and/or HIV-2 viruses, present in a subject, which comprises:

- 10 a) obtaining a sample of a body fluid, preferably a blood sample comprising antigen presenting cells, from a subject,
- b) adding to said sample a ligand and/or an anti-ligand as mentioned above,
- 15 c) detecting the cross-reaction between said ligand and/or said anti-ligand and the specific peptide, and
- d) determining whether the peptide corresponds to a receptor or an inactive receptor and diagnosing thereby a predisposition or a resistance to the disorder according to the type of the peptide present in the body fluid of the subject.

20 **[0091]** The present invention further relates to a diagnostic and/or dosage device comprising the peptide, the nucleic acid molecule, the nucleic acid probe, the ligand and anti-ligand as mentioned above, the known "natural ligands" their portions (such as primer, probes, epitopes, ...) and/or a mixture thereof, being possibly labelled with a detectable marker.

25 **[0092]** Further, the present invention relates to a diagnostic and/or dosage device as mentioned above, characterised in that it comprises the reactants for the detection and/or dosage of antigens, antibodies or nucleic acid sequences through a method selected from the group consisting of in situ hybridisation, hybridisation or recognition by marked specific antibodies, specially ELISA® (Enzyme Linked Immunosorbent Assay) or RIA® (Radio Immunoassay), methods on filter, on a solid support, in solution, in "sandwich", on gel, by Dot blot hybridisation, by Northern blot hybridisation, by Southern blot hybridisation, by isotopic or non-isotopic labelling (such as immunofluorescence or biotinylation) , by

30 a technique of cold probes, by genetic amplification, particularly PCR, LCR, NASBA or CPR, by a double immunodiffusion, by a counter-immunoelectrophoresis, by haemagglutination and/or a mixture thereof.

[0093] The present invention further relates to a method of preparing peptides as mentioned above, which comprises:

- 35 a) constructing a vector adapted for expression in a cell which comprises the regulatory elements necessary for the expression of nucleic acid molecules in the cell operatively linked to nucleic acid molecule encoding said peptide so as to permit expression thereof, wherein the cell is preferably selected from the group consisting of bacterial cells, yeast cells, insect cells and mammalian cells,
- b) inserting the vector of step a) in a suitable host cell,
- 40 c) incubating the cell of step b) under conditions allowing the expression of the peptide according to the invention,
- d) recovering the peptide so obtained, and possibly
- 45 e) purifying the peptide so recovered.

The present invention relates also to the use of the pharmaceutical composition as mentioned above, for the preparation of a medicament in the treatment of a disease chosen from the group consisting of inflammatory diseases, including rheumatoid arthritis, glomerulonephritis, asthma, idiopathic pulmonary fibrosis and psoriasis, viral infections including

50 infections by Human Immunodeficiency Viruses 1 and 2 (HIV-1 and 2), cancer including leukaemia, atherosclerosis and/or autoimmune disorders.

[0094] The present invention is related to a peptide having at least an amino acid sequence which presents more than 80%, advantageously more than 90%, preferably more than 95%, homology with the amino acid sequence as represented in SEQ ID NO. 1.

55 **[0095]** Preferably, said peptide has also at least an amino acid sequence which presents more than 80%, advantageously more than 90%, preferably more than 95%, homology with the amino acid sequence as represented in SEQ ID NO. 2.

[0096] According to another embodiment of the present invention, the peptide has at least an amino acid sequence

which presents more than 80%, advantageously more than 90%, preferably more than 95%, homology with the amino acid sequence as represented in SEQ ID NO. 3.

[0097] The present invention is also related to the amino acid sequence of SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3 or a portion thereof (represented in the fig. 1).

5 [0098] A "portion of an amino acid sequence" means one or more amino acid segments having the same or improved binding properties of the whole peptide according to the invention. Said portion could be an epitope which is specifically binded by a ligand of the peptide which could be a known "natural ligand" of said peptide, an agonist or an analog of said ligand, or an inhibitor capable of competitively inhibiting the binding of said ligand to the peptide (including the antagonists of said ligand to the peptide).

10 [0099] Specific examples of said portions of amino acid sequence and their preparation process are described in the publication of Rucker J. et al. (Cell, Vol. 87, pp. 437-446 (1996)) incorporated herein by reference.

[0100] According to the invention, said portion of the amino acid sequence of the peptide according to the invention comprises the N-terminus segment and the first extracellular loop of the peptide.

15 [0101] Therefore, according to the invention, the amino acid sequence as represented in SEQ ID NO. 1 is the common amino acid sequence of SEQ ID NO. 2 and of SEQ ID NO. 3 (see also figure 1). Therefore, a first industrial application of said amino acid sequence is the identification of the homology between said amino acid sequence and the screening of various mutants encoding a different amino acid sequence than the one previously described, and the identification of various types of patient which may present a predisposition or a resistance to the disorders described in the following specification.

20 [0102] Preferably, the peptide according to the invention or a portion thereof is an active CC-chemokine receptor.

[0103] Advantageously, the CC-chemokine receptor according to the invention is stimulated by the MIP-1 β chemokine at a concentration less or equal to 10 nm, and is advantageously also stimulated by the MIP-1 α or RANTES chemokines. However, said chemokine receptor is not stimulated by the MCP-1, MCP-2, MCP-3, IL-8 and GRO α chemokines.

25 [0104] In addition, the peptide according to the invention or a portion thereof is also a receptor of HIV viruses or a portion of said HIV viruses.

[0105] It is meant by "HIV viruses", HIV-1 or HIV-2 and all the various strains of HIV viruses which are involved in the development of AIDS. It is meant by a "a portion of HIV viruses", any epitope of said viruses which is able to interact specifically with said receptor. Among said portions of viruses which may be involved in the interaction with the peptide according to the invention, are peptides encoded by the ENV and GAG viruses genes.

30 [0106] Preferably, said portion of HIV viruses is the glycopeptide gp120/160 (membrane-bound gp160 or the free gp derived therefrom) or a portion thereof.

[0107] It is meant by a "portion of the glycopeptide gp120/160" any epitope, preferably an immuno-dominant epitope, of said glycopeptide which may interact specifically with the peptide according to the invention, such as for instance the V3 loop (third hypervariable domain).

35 [0108] According to another embodiment of the present invention, the peptide according to the invention is an inactive CC-chemokine receptor. An example of such inactive CC-chemokine receptor is encoded by the amino acid sequence as represented in SEQ ID NO. 2.

[0109] It is meant by an "inactive CC-chemokine receptor" a receptor which is not stimulated by any known CC-chemokine, especially the MIP-1 β , MIP-1 α or RANTES chemokines.

40 [0110] The peptide represented in SEQ ID NO. 3 according to the invention is an inactive receptor which is not a receptor of HIV viruses or of a portion of said HIV viruses, which means that said inactive receptor does not allow the entry of said HIV viruses into a cell which presents at its surface said inactive receptor.

[0111] Advantageously, the peptide according to the invention is a human receptor.

45 [0112] The present invention concerns also the nucleic acid molecule having more than 80%, preferably more than 90%, homology with one of the nucleic acid sequences of SEQ ID NO. 1, SEQ ID NO. 2 and SEQ ID NO. 3 shown in the figures 1.

[0113] Preferably, said nucleic acid molecule has at least the nucleic acid sequence shown in SEQ ID NO. 1, SEQ ID NO. 2 or SEQ ID NO. 3 of figure 1 or a portion thereof.

50 [0114] It is meant by a "portion of said nucleic acid molecule" any nucleic acid sequence of more than 15 nucleotides which could be used in order to detect and/or reconstitute said nucleic acid molecule or its complementary strand. Such portion could be a probe or a primer which could be used in genetic amplification using the PCR, LCR, NASBA or CPR techniques for instance.

55 [0115] The present invention concerns more specifically the nucleic acid molecules encoding the peptide according to the invention. Said nucleic acid molecules are RNA or DNA molecules such as a cDNA molecule or a genomic DNA molecule.

[0116] The present invention is also related to a vector comprising the nucleic acid molecule according to the invention. Preferably, said vector is adapted for expression in a cell and comprises the regulatory elements necessary for

expressing the amino acid molecule in said cell operatively linked to the nucleic acid sequence according to the invention as to permit expression thereof.

[0117] Preferably, said cell is chosen among the group consisting of bacterial cells, yeast cells, insect cells or mammalian cells. The vector according to the invention is a plasmid, preferably a pcDNA3 plasmid, or a virus, preferably a baculovirus, an adenovirus or a semliki forest virus.

[0118] The present invention concerns also the cell, preferably a mammalian cell, such as a CHO-K1 or a HEK293 cell, transformed by the vector according to the invention. Advantageously, said cell is non neuronal in origin and is chosen among the group consisting of CHO-K1, HEK293, BHK21, COS-7 cells.

[0119] The present invention also concerns the cell (preferably a mammalian cell such as a CHO-K1 cell) transformed by the vector according to the invention and by another vector encoding a protein enhancing the functional response in said cell. Advantageously, said protein is the $\alpha 15$ or $\alpha 16$ (G protein, α subunit). Advantageously, said cell is the cell CHO-K1-pEFIN hCCR5-1/16.

[0120] The present invention is also related to a nucleic acid probe comprising a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridising with a unique sequence included within the sequence of the nucleic acid molecule according to the invention. Said nucleic acid probe may be a DNA or a RNA.

[0121] The invention concerns also an antisense oligonucleotide having a sequence capable of specifically hybridising to an mRNA molecule encoding the peptide according to the invention so as to prevent translation of said mRNA molecule or an antisense oligonucleotide having a sequence capable of specifically hybridising to the cDNA molecule encoding the peptide according to the invention.

[0122] Said antisense oligonucleotide may comprise chemical analogs of nucleotide or substances which inactivate mRNA, or be included in an RNA molecule endowed with ribozyme activity.

[0123] Another aspect of the present invention concerns a ligand or an anti-ligand (preferably an antibody) other than known "natural ligands", which are chosen among the group consisting of the MIP-1 β , MIP-1 α or RANTES chemokines, HIV viruses or a portion of said HIV viruses, wherein said ligand is capable of binding to the receptor according to the invention and wherein said anti-ligand is capable of (preferably competitively) inhibiting the binding of said known "natural ligand" or the ligand according to the invention to the peptide according to the invention.

[0124] The exclusion in the above identified definition of known chemokines, HIV viruses or a portion of said HIV viruses, does not include variants of said "natural" viruses or said "natural" portion which may be obtained for instance by genetic engineering and which may mimic the interaction of said viruses and portion of said viruses to the peptide according to the invention.

[0125] Advantageously, said antibody is a monoclonal antibody which is preferably directed to an epitope of the peptide according to the invention and present on the surface of a cell expressing said peptide.

[0126] Preferably, said antibody is produced by the hybridoma cell AchCCR5-SAB1A7.

[0127] The invention concerns also the pharmaceutical composition comprising either an effective amount of the peptide according to the invention (in order to delude the HIV virus from the natural peptide present at the surface of a mammalian cell and stop the infection of said mammalian cell by the HIV virus), or an effective amount of the above identified described ligand and/or anti-ligand, or an effective amount of oligonucleotide according to the invention, effective to decrease the activity of said peptide by passing through a cell membrane and binding specifically with mRNA encoding the peptide according to the invention in the cell so as to prevent its translation. The pharmaceutical composition comprises also a pharmaceutically acceptable carrier, preferably capable of passing through said-cell membrane.

[0128] Preferably, in said pharmaceutical composition, the oligonucleotide is coupled to a substance, such as a ribozyme, which inactivates mRNA encoding the peptide according to the invention.

[0129] Preferably, the pharmaceutically acceptable carrier comprises a structure which binds to a receptor on a cell capable of being taken up by cell after binding to the structure. The structure of the pharmaceutically acceptable carrier in said pharmaceutical composition is capable of binding to a receptor which is specific for a selected cell type.

[0130] The present invention concerns also a transgenic non human mammal overexpressing (or expressing ectopically) the nucleic acid molecule encoding the peptide according to the invention.

[0131] The present invention also concerns a transgenic non human mammal comprising an homologous recombination knockout of the native peptide according to the invention.

[0132] According to a preferred embodiment of the invention, the transgenic non human mammal whose genome comprises antisense nucleic acid complementary to the nucleic acid according to the invention is so placed as to be transcribed into antisense mRNA which is complementary to the mRNA encoding the peptide according to the invention and which hybridises to mRNA encoding said peptide, thereby reducing its translation. Preferably, the transgenic non human mammal according to the invention comprises a nucleic acid molecule encoding the peptide according to the invention and comprises additionally an inducible promoter or a tissue specific regulatory element.

[0133] Preferably, the transgenic non human mammal is a mouse.

[0134] The invention relates to a method for determining whether a ligand can be specifically bound to the peptide

according to the invention, which comprises contacting a cell transfected with a vector expressing the nucleic acid molecule encoding said peptide with the ligand under conditions permitting binding of ligand to such peptide and detecting the presence of any such ligand bound Specifically to said peptide, thereby determining whether the ligand binds specifically to said peptide.

[0135] The invention relates to a method for determining whether a ligand can specifically bind to a peptide according to the invention, which comprises preparing a cell extract from cells transfected with a vector expressing the nucleic acid molecule encoding said peptide, isolating a membrane fraction from the cell extract, contacting the ligand with the membrane fraction under conditions permitting binding of the ligand to such peptide and detecting the presence of a ligand bound to said peptide, thereby determining whether the compound is capable of specifically binding to said peptide. Preferably, said method is used when the ligand is not previously known.

[0136] The invention relates to a method for determining whether a ligand is an agonist of the peptide according to the invention, which comprises contacting a cell transfected with a vector expressing the nucleic acid molecule encoding said peptide with the ligand under conditions permitting the activation of a functional peptide response from the cell and detecting by means of a bio-assay, such as a modification in a second messenger concentration (preferably calcium ions or inositol phosphates such as IP_3) or a modification in the cellular metabolism (preferably determined by the acidification rate of the culture medium), an increase in the peptide activity, thereby determining whether the ligand is a peptide agonist.

[0137] As used herein the term agonist refers to a ligand that activates an intracellular response when it binds to a receptor.

[0138] The invention relates to a method for determining whether a ligand is an agonist of the peptide according to the invention, which comprises preparing a cell extract from cells transfected with a vector expressing the nucleic acid molecule encoding said peptide, isolating a membrane fraction with the ligand under conditions permitting the activation of a functional peptide response and detecting by means of a bio-assay, such as a modification in the production of a second messenger (preferably inositol phosphates such as IP_3), an increase in the peptide activity, thereby determining whether the ligand is an antagonist of the peptide according to the invention, which comprises contacting a cell transfected with a vector expressing the nucleic acid molecule encoding said peptide with the ligand in the presence of a known peptide agonist, under conditions permitting the activation of a functional peptide response and detecting by means of a bio-assay, such as a modification in second messenger concentration (preferably calcium ions or inositol phosphates such as IP_3) or a modification in the cellular metabolism (preferably determined by the acidification rate of the culture medium), a decrease in the peptide activity, thereby determining whether the ligand is a peptide antagonist.

[0139] As used herein the term antagonist is a ligand which competitively binds to a receptor at the same site as an agonist, but does not activate an intracellular response initiated by a receptor.

[0140] The present invention relates to a method for determining whether a ligand is an antagonist of the peptide according to the invention, which comprises preparing a cell extract from cells transfected with an expressing the nucleic acid molecule encoding said peptide, isolating a membrane fraction from the cells extract, contacting the membrane fraction with the ligand in the presence of a known peptide agonist, under conditions permitting the activation of a functional peptide response and detecting by means of a bio-assay, such as a modification in the production of a second messenger, a decrease in the peptide activity, thereby determining whether the ligand is a peptide antagonist.

[0141] Preferably, the second messenger assay comprises measurement of calcium ions or inositol phosphates such as IP_3 .

[0142] Preferably, the cell used in said method is a mammalian cell non neuronal in origin, such as CHO-K1, HEK293, BHK21, COS-7 cells.

[0143] In said method, the ligand is not previously known.

[0144] The invention is also related to the ligand isolated and detected by any of the preceding methods.

[0145] The present invention concerns also the pharmaceutical composition which comprises an effective amount of an agonist or an antagonist of the peptide according to the invention, effective to reduce the activity of said peptide and a pharmaceutically acceptable carrier.

[0146] It is meant by "an agonist or an antagonist of the peptide, according to the invention", all the agonists or antagonists of the known "natural ligand" of the peptide as above described.

[0147] Therefore, the previously described methods may be used for the screening of drugs to identify drugs which specifically bind to the peptide according to the invention.

[0148] The invention is also related to the drugs isolated and detected by any of these methods.

[0149] The present invention concerns also a pharmaceutical composition comprising said drugs and a pharmaceutically acceptable carrier.

[0150] The invention is also related to a method of detecting expression of a peptide according to the invention by detecting the presence of mRNA coding for a peptide, which comprises obtaining total RNA or total mRNA from the cell and contacting the RNA or mRNA so obtained with the nucleic acid probe according to the invention under hybrid-

ising conditions and detecting the presence of mRNA hybridised to the probe, thereby detecting the expression of the peptide by the cell.

[0151] Said hybridisation conditions are stringent conditions.

[0152] The present invention concerns also the use of the pharmaceutical composition according to the invention for the treatment and/or prevention of inflammatory diseases, including rheumatoid arthritis, glomerulonephritis, asthma, idiopathic pulmonary fibrosis and psoriasis, viral infections including Human Immunodeficiency Viruses 1 and 2 (HIV-1 and 2), cancer including leukaemia, atherosclerosis and/or auto-immune disorders.

[0153] The present invention concerns also a method for diagnosing a predisposition or a resistance to a disorder associated with the activity of the peptide according to the invention and/or associated with infectious agents such as HIV viruses in a subject. Said method comprises :

- a) obtaining nucleic acid molecules encoding the peptide according to the invention from the cells of the subject;
- b) possibly performing a restriction digest of said nucleic acid molecules with a panel of restriction enzymes;
- c) possibly electrophoretically separating the resulting nucleic acid fragments on a sized gel;
- d) contacting the resulting gel or the obtained nucleic acid molecule with a nucleic acid probe labelled with a detectable marker and capable of specifically hybridising to said nucleic acid molecule (said hybridisation being made in stringent hybridisation conditions);
- e) detecting labelled bands or the in situ nucleic acid molecules which have hybridised to the said nucleic acid molecule labelled with a detectable marker to create a unique band pattern or an in situ marking specific to the subject;
- f) preparing other nucleic acid molecules encoding the peptide according to the invention obtained from the cells of other patients for diagnosis by step a-e; and
- g) comparing the unique band pattern specific to the nucleic acid molecule of subjects suffering from the disorder from step e and the nucleic acid molecule obtained for diagnosis from step f to determine whether the patterns are the same or different and to diagnose thereby a predisposition or a resistance to the disorder if the patterns are the same or different.

[0154] The present invention is also related to a method for diagnosing a predisposition or a resistance to a disorder associated with the activity of a specific allele of the peptide according to the invention or the presence of said peptide at the surface of cells and/or associated with infectious agents such as HIV viruses present in a subject. Said method comprises :

- a) obtaining a sample of a body fluid, preferably a blood sample comprising antigen presenting cells, from a subject;
- b) adding to said sample a ligand and/or an anti-ligand according to the invention;
- c) detecting the cross-reaction between said ligand and/or said anti-ligand and the specific peptide according to the invention; and
- d) determining whether the peptide corresponds to a receptor or an inactive receptor according to the invention and diagnosing thereby a predisposition or a resistance to the disorder according to the type of the peptide present in the body fluid of the subject.

[0155] The present invention concerns also a diagnostic and/or dosage device, preferably a kit, comprising the peptides, the nucleic acid molecules, the nucleic acid probes, the ligands and/or the anti-ligands according to the invention, their portions (such as primers, probes, epitopes, ...) or a mixture thereof, being possibly labelled with a detectable marker.

[0156] Said diagnostic and/or dosage device comprises also the reactants for the detection and/or the dosage of antigens, antibodies or nucleic acid sequences through a method selected from the group consisting of in situ hybridisation, hybridisation or recognition by marked specific antibodies, specially ELISA® (Enzyme Linked Immunosorbent Assay) or RIA® (Radio Immunoassay), methods on filter, on a solid support, in solution, in "sandwich", on gel, by Dot blot hybridisation, by Northern blot hybridisation, by Southern blot hybridisation, by isotopic or non-isotopic labelling (such as immunofluorescence or biotinylation), by a technique of cold probes, by genetic amplification, particularly PCR, LCR, NASBA or CPR, by a double immunodiffusion, by a counter-immunoelectrophoresis, by haemagglutination and/or a mixture thereof.

[0157] A last aspect of the present invention concerns a method of preparing peptides according to the invention, which comprises :

- a) constructing a vector adapted for expression in a cell which comprises the regulatory elements necessary for the expression of nucleic acid molecules in the cell operatively linked to nucleic acid molecule encoding said peptide so as to permit expression thereof, wherein the cell is preferably selected from the group consisting of

- bacterial cells, yeast cells, insect cells and mammalian cells;
 b) inserting the vector of step a in a suitable host cell;
 c) incubating the cell of step b under conditions allowing the expression of the peptide according to the invention;
 d) recovering the peptide so obtained; and
 e) purifying the peptide so recovered, thereby preparing an isolated peptide according to the invention.

[0158] The deposits of micro-organisms AchCCR5-SAB1A7 and CHO-K1-pEFIN hCCR5-1/16 were made according to the Budapest Treaty in the Belgium Coordinated Collection of Micro-organisms (BCCM), Laboratorium voor Moleculaire Biologie (LMBP), Universiteit Gent, K. L. Ledeganckstraat 35, 3-9000 GENT, BELGIUM.

Short description of the drawings.

[0159]

- The figure 1 represents the primary structure of the peptides according to the invention.
 The figure 2 represents the amino acids sequence of the active human CCR5 chemokine receptor according to the invention aligned with that of the human CCR1, CCR2b, CCR3 and CCR4 receptors. Amino acids identical with the active CCR5 sequence are boxed.
 The figure 3 shows the chromosomal organisation of the human CCR2 and CCR5 chemokine receptor genes.
 The figure 4 shows the functional expression of the human active CCR5 receptor in a CHO-K1 cell line.
 The figure 5 represents the distribution of mRNA encoding the CCR5 receptor in a panel of human cell lines of haematopoietic origin.
 The figure 6 represents the structure of the mutant form of human CCR5 receptor.
 The figure 7 represents the quantification of ENV proteins-mediated fusion by luciferase assays.
 The figure 8 represents genotyping of individuals by PCR and segregation of the CCR5 alleles in CEPH families.
 The figure 9 represents the FACS analysis of sera anti-CCR5 on a CCR5-CHO cell line according to the invention.
 The figure 10 represents the inhibition of HIV infectivity with anti-CCR5 antibodies.

Detailed description of the invention.

1. EXPERIMENTALS

Materials

[0160] Recombinant human chemokines, including MCP-1, MIP-1 α , MIP-1 β , RANTES, IL-8 and GRO α were obtained from R & D Systems (London, UK). [¹²⁵I]MIP-1 α (specific activity, 2200 Ci/mmol) was obtained from Dupont NEN (Brussels, Belgium). Chemokines obtained from R & D Systems were reported by the supplier as >97 % pure on SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) and biologically active on a bioassay specific for each ligand. The lyophilised chemokines were dissolved as a 100 μ g/ml solution in a sterile phosphate-buffered saline (PBS) and this stock solution was stored at -20° C in aliquots. Chemokines were diluted to the working concentration immediately before use. All cell lines used in the present study were obtained from the ATCC (Rockville, MD, USA).

Cloning and sequencing

[0161] The mouse MOP020 clone was obtained by low stringency polymerase chain reaction, as described previously [24, 34], using genomic DNA as template. A human genomic DNA library (Stratagene, La Jolla, CA) constructed in the lambda DASH vector was screened at low stringency [39] with the MOP020 (511 bp) probe. The positive clones were purified to homogeneity and analysed by Southern blotting. The restriction map of the locus was determined and a relevant XbaI fragment of 4,400 bp was subcloned in pBluescript SK+ (Stratagene). Sequencing was performed on both strands after subcloning in M13mp derivatives, using fluorescent primers and an automated DNA sequencer (Applied Biosystem 370A). Sequence handling and data analysis was carried out using the DNASIS/PROSIS software (Hitachi), and the GCG software package (Genetics Computer Group, Wisconsin).

Expression in cell lines

[0162] The entire coding region was amplified by PCR as a 1056 bp fragment, using primers including respectively the *Bam*HI and *Xba*I recognition sequences, and cloned after restriction in the corresponding sites of the eukaryotic

expression vector pcDNA3 (Invitrogen, San Diego, CA). The resulting construct was verified by sequencing, and transfected in CHO-K1 cells as described [35]. Two days after transfection, selection for stably transfected cell lines was initiated by the addition of 400 µg/ml G418 (Gibco), and resistant clones were isolated at day 10. CHO-K1 cells were cultured using Ham's F12 medium, as previously described [35, 11]. The expression of the active CCR5 receptor in the various cell clones was evaluated by measuring the specific transcript level by Northern blotting, on total RNA prepared from the cells (see below).

Binding Assays

[0163] Stably transfected CHO-K1 cells expressing the active CCR5 receptor were grown to confluence and detached from culture dishes by incubation in phosphate-buffered saline (PBS) supplemented with 1 mM EDTA. Cells were collected by low speed centrifugation and counted in a Neubaer cell. Binding assays were performed in polyethylene minisorp tubes (Nunc) in a final volume of 200 µl PBS containing 0.2 % bovine serum albumin (BSA) and 10^6 cells, in presence of [125 I]-MIP-1 α . Non specific binding was determined by addition of 10 nM unlabelled MIP-1 α . The concentration of labelled ligand was 0.4 nM (around 100 000 cpm per tube). The incubation was carried out for 2 hours at 4 °C, and was stopped by the rapid addition of 4 ml ice-cold buffer, and immediate collection of cells by vacuum filtration through GF/B glass fiber filters (Whatmann) pre-soaked in 0.5 % polyethylenimine (Sigma). Filters were washed three times with 4 ml ice-cold buffer and counted in a gamma counter.

Biological activity

[0164] The CHO-K1 cell lines stably transfected with the pcDNA3/CCR5 construct or wild type CHO-K1 cells (used as controls) were plated onto the membrane of Transwell cell capsules (Molecular Devices), at a density of 2.5×10^5 cells/well in Ham's F12 medium. The next day, the capsules were transferred in a microphysiometer (Cytosensor, Molecular Devices), and the cells were allowed to equilibrate for approximately two hours by perfusion of 1 mM phosphate-buffered (pH 7.4) RPMI-1640 medium containing 0.2 % BSA. Cells were then exposed to various chemokines diluted in the same medium, for a 2 min duration. Acidification rates were measured at one minute intervals.

Northern blotting

[0165] Total RNA was isolated from transfected CHO-K1 cell lines, from a panel of human cell lines of haematopoietic origin and from a panel of dog tissues, using the RNeasy kit (Qiagen). RNA samples (10 µg per lane) were denatured in presence of glyoxal [26], fractionated on a 1 % agarose gel in a 10 mM phosphate buffer (pH 7.0), and transferred to nylon membranes (Pall Biodyne A, Glen Cove, NY) as described [42]. After baking, the blots were prehybridised for 4h at 42° C in a solution consisting of 50 % formamide, 5x Denhardt solution (1x Denhardt : 0.02 % Ficoll, 0.02 % polyvinylpyrrolidone, 0.02 % BSA), 5x SSPE (1x SSPE: 0.18 M NaCl, 10 mM Na phosphate, 1 mM EDTA pH 8.3), 0.3 % Sodium Dodecyl Sulphate (SDS), 250 µg per ml denatured DNA from herring testes. DNA probes were (α^{32} P)-labelled by random priming [14]. Hybridisations were carried out for 12h at 42° C in the same solution containing 10 % (wt/vol) dextran sulphate and the heat denatured probe. Filters were washed up to 0.1x SSC (1x SSC: 150 mM NaCl, 15 mM Na Citrate pH 7.0), 0.1 % SDS at 60° C and autoradiographed at - 70° C using Amersham β -max films.

2. RESULTS AND DISCUSSION

Cloning and structural analysis

[0166] The sequence homology characterising genes encoding G protein-coupled receptors has allowed the cloning by low stringency polymerase chain reaction (PCR) of new members of this gene family [24, 34]. One of the clones amplified from mouse genomic DNA, named MOP020 presented strong similarities with characterised chemokine receptors, sharing 80 % identity with the MCP-1 receptor (CCR2) [8], 65 % identity with the MIP-1 α /RANTES receptor (CCR1) [31], and 51 % identity with IL-8 receptors [20, 30]. The clone was used as a probe to screen a human genomic library. A total of 16 lambda phage clones were isolated. It was inferred from the restriction pattern of each clone and from partial sequence data that all clones were belonging to a single contig in which two different coding sequences were included. One of the coding sequences was identical to the reported cDNA encoding the CCR2 receptor [8, 44]. A 4.400 pb XbaI fragment of a representative clone containing the second region of hybridisation was subcloned in pBluescript SK+. Sequencing revealed a novel gene, tentatively named CCR5, sharing 84 % identity with the MOP020 probe, suggesting that MOP020 is the mouse ortholog of CCR5. MOP020 does not correspond to any of the three mouse chemokine receptor genes cloned recently [16], demonstrating the existence of a fourth murine chemokine receptor.

[0167] The sequence of CCR5 revealed a single open reading frame of 352 codons encoding a protein of 40,600 Da. The sequence surrounding the proposed initiation codon is in agreement with the consensus as described by Kozak [22], since the nucleotide in -3 is a purine. The hydropathy profile of the deduced amino acid sequence is consistent with the existence of 7 transmembrane segments. Alignment of the CCR5 amino acid sequence with that of other functionally characterised human CC-chemokine receptors is represented in figure 2. The highest similarity is found with the CCR2 receptor [8] that shares 75.8 % identical residues. There is also 56.3 % identity with the CCR1 receptor [31], 58.4 % with the CCR3 [10], and 49.1% with the CCR4 [37]. CCR5 represents therefore a new member of the CC-chemokine receptor group [30]. Like the related CCR1 and IL-8 receptors [20, 29, 31, 16] the coding region of CCR5 appears as intronless. From our partial sequencing data, the CCR2 gene is also devoid of intron in the first two thirds of its coding sequence.

[0168] Sequence similarities within the chemokine receptor family are higher in the transmembrane-spanning domains, and in intracellular loops. As an example, the identity score between CCR5 and CCR2 goes up to 92% when considering the transmembrane segments only. Lower similarities are found in the N-terminal extracellular domain, and in the extracellular loops. The N-terminal domain of the IL-8 and CCR2 receptors has been shown to be essential for interaction with the ligand [19, 18]. The variability of this region among CC-chemokine receptors presumably contributes to the specificity towards the various ligands of the family.

[0169] A single potential site for N-linked glycosylation was identified in the third extracellular loop of CCR5 (figure 1). No glycosylation site was found in the N-terminal domain of the receptor, where most G protein-coupled receptors are glycosylated. The other chemokine receptors CCR1 and CCR2 present such an N-linked glycosylation site in their N-terminal domain [31, 8]. By contrast, the CCR3 receptor [10] does not display glycosylation sites neither in the N-terminus, nor in extracellular loops. The active CCR5 receptor has four cysteines in its extracellular segments, and all four are conserved in the other CC- and CXC-chemokine receptors (figure 2). The cysteines located in the first and second extracellular loops are present in most G protein-coupled receptors, and are believed to form a disulphide bridge stabilising the receptor structure [41]. The two other cysteines, in the N-terminal segment, and in the third extracellular loop could similarly form a stabilising bridge specific to the chemokine receptor family. The intracellular domains of CCR5 do not include potential sites for phosphorylation by protein kinase C (PKC) or protein kinase A. PKC sites, involved in heterologous desensitisation are frequent in the third intracellular loop and C-terminus of G protein-coupled receptors. CCR1 is also devoid of PKC sites. In contrast, all CC-chemokine receptors, are rich in serine and threonine residues in the C-terminal domain. These residues represent potential phosphorylation sites by the family of G protein-coupled receptor kinases, and are probably involved in homologous desensitisation [41]. Five of these S/T residues are perfectly aligned in all five receptors (figure 2).

Physical linkage of the CCR5 and CCR2 genes

[0170] As stated above, the 16 clones isolated with the MOP020 probe corresponded to a single contig containing the CCR5 and CCR2 genes. The organisation of this contig was investigated in order to characterise the physical linkage of the two receptor genes in the human genome. A combination of restriction mapping, Southern blotting, fragment subcloning and partial sequencing allowed to determine the respective borders and overlaps of all clones. Out of the 16 clones, 9 turned out to be characterised by a specific restriction map, and their organisation is depicted in figure 3. Four of these clones (#11, 18, 21, 22) contained the CCR2 gene alone, four clones (# 7, 13, 15, 16) contained the ChemR13 gene alone and one clone (#9) contains part of both coding sequences. The CCR2 and CCR5 genes are organised in tandem, CCR5 being located downstream of CCR2. The distance separating CCR2 and CCR5 open reading frames is 17.5 kb. The chromosomal localisation of the tandem is presently unknown. Other chemokine receptors have however been located in the human genome: the CCR1 gene was localised by fluorescence in situ hybridisation to the p21 region of human chromosome 3 [16]. The two IL-8 receptor genes, and their pseudogene have been shown to be clustered on the human 2q34-q35 region [1].

Functional expression and pharmacology of the active CCR5 receptor

[0171] Stable CHO-K1 cell lines expressing the active CCR5 receptor were established and were screened on the basis of the level of CCR5 transcripts as determined by Northern blotting. Three clones were selected and tested for biological responses in a microphysiometer, using various CC- and CXC-chemokines as potential agonists. Wild type CHO-K1 cells were used as control to ensure that the observed responses were specific for the transfected receptor, and did not result from the activation of endogenous receptors. The microphysiometer allows the real time detection of receptor activation, by measuring the modifications of cell metabolism resulting from the stimulation of intracellular cascades [33]. Several studies have already demonstrated the potential of microphysiometry in the field of chemokine receptors. Modifications of metabolic activity in human monocytes, in response CC-chemokines, were monitored using this system [43]. Similarly, changes in the acidification rate of THP-1 cells (a human monocytic cell line) in response

to MCP-1 and MCP-3 have been measured [36]. The estimation of the EC_{50} for both proteins, using this procedure, was in agreement with the values obtained by monitoring the intracellular calcium in other studies [8, 15].

[0172] Ligands belonging to the CC- and CXC-chemokine classes were tested on the CCR5 transfected CHO-K1 cells. Whereas MIP-1 α , MIP-1 β and RANTES were found to be potent activators of the new receptor (figure 4), the CC-chemokines MCP-1, MCP-2 and MCP-3, and the CXC-chemokines GRO α and IL-8 had no effect on the metabolic activity, even at the highest concentrations tested (30 nM). The biological activity of one of the chemokines inducing no response on CCR5 (IL-8) could be demonstrated on a CHO-K1 cell line transfected with the IL-8A interleukin receptor (Mollereau et al., 1993) : IL-8 produced a 160 % increase in metabolic activity as determined using the microphysiometer. The biological activity of the MCP-2 and MCP-3 preparations as provided by J. Van Damme have been widely documented [2, 40]. MIP-1 α , MIP-1 β and RANTES were tested on the wild type CHO-K1 cells, at a 30 nM-concentration, and none of them induced a metabolic response. On the CCR5 transfected CHO-K1 cell line, all three active ligands (MIP-1 α , MIP-1 β and RANTES) caused a rapid increase in acidification rate, reaching a maximum by the second or third minute after perfusion of the ligand. The acidification rate returned to basal level within 10 minutes. The timing of the cellular response is similar to that observed for chemokines on their natural receptors in human monocytes [43]. When agonists were applied repeatedly to the same cells, the response was strongly reduced as compared to the first stimulation, suggesting the desensitisation of the receptor. All measurements were therefore obtained on the first stimulation of each capsule.

[0173] The concentration-effect relation was evaluated for the three active ligands in the 0.3 to 30 nM range (figure 3B and C). The rank order of potency was MIP-1 α > MIP-1 β = RANTES. At 30 nM concentrations, the effect of MIP-1 α appeared to saturate (at 156 % of baseline level) while MIP-1 β and RANTES were still in the ascending phase. Higher concentrations of chemokines could however not be used. The EC_{50} was estimated around 3 nM for MIP-1 α . The concentrations necessary for obtaining a biological response as determined by using the microphysiometer are in the same range as those measured by intracellular calcium mobilisation for the CCR1 [31], the CCR2A and B [8], and the CCR3 [10] receptors. The ligand specificity of CCR5 is similar to that reported for CCR3 [10]. CCR3 was described as the first cloned receptor responding to MIP-1 β . However, MIP-1 β at 10 nM elicits a significant effect on the CCR5, while the same concentration is without effect on the CCR3 transfected cells [10]. These data suggest that CCR5 could be a physiological receptor for MIP-1 β .

[0174] Binding experiments using [125 I]-human MIP-1 α as ligand did not allow to demonstrate specific binding to CCR5 expressing CHO-K1 cells, using as much as 0.4 nM radioligand and 1 million transfected cells per tube. Failure to obtain binding data could be attributed to a relatively low affinity of the receptor for MIP-1 α .

Northern blotting analysis

[0175] Northern blotting performed on a panel of dog tissues did not allow to detect transcripts for CCR5. Given the role of the chemokine receptor family in mediating chemoattraction and activation of various classes of cells involved in inflammatory and immune responses, the probe was also used to detect specific transcripts in a panel of human cell lines of haematopoietic origin (figure 5). The panel included lymphoblastic (Raji) and T lymphoblastic (Jurkat) cell lines, promyeloblastic (KG-1A) and promyelocytic (HL-60) cell lines, a monocytic (THP-1) cell line, an erythroleukemia (HEL 92.1.7) cell line, a megakaryoblastic (MEG-01) cell line, and a myelogenous leukaemia (K-562) cell line. Human peripheral blood mononuclear cells (PBMC), including mature monocytes and lymphocytes, were also tested. CCR5 transcripts (4.4 kb) could be detected only in the KG-1A promyeloblastic cell line, but were not found in the promyelocytic cell line HL-60, in PBMC, or in any of the other cell lines tested. These results suggest that the active CCR5 receptor could be expressed in precursors of the granulocytic lineage. CC-chemokines have been reported to stimulate mature granulocytes [27, 38, 23, 2]. However, recent data have also demonstrated a role of CC- and CXC-chemokines in the regulation of mouse and human myeloid progenitor cell proliferation [6, 7].

[0176] CCR5 was shown to respond to MIP-1 α , MIP-1 β and RANTES, the three chemokines identified as the major HIV-suppressive factors produced by CD8 $^{+}$ T cells [9], and released in higher amounts by CD4 $^{+}$ T lymphocytes from uninfected but multiply exposed individuals [51]. CCR5 represents a major co-receptor for macrophage-tropic (M-tropic) HIV-1 primary isolates and strains [45, 50]. M-tropic strains predominate during the asymptomatic phase of the disease in infected individuals, and are considered as responsible for HIV-1 transmission. Strains adapted for growth in transformed T-cell lines (T-tropic strains) use as a co-receptor LESTR (or fusin) [50], an orphan receptor also belonging to the chemokine receptor family, but not yet characterised functionally [21, 52, 53]. Dual-tropic viruses, which may represent transitional forms of the virus in late stages of infection [54] are shown to use both CCR5 and LESTR as co-receptors, as well as the CC-chemokine receptors CCR2b and CCR3 [47]. The broad spectrum of co-receptor usage of dual-tropic viruses suggests that within infected individuals, the virus may evolve at least in part from selection by a variety of co-receptors expressed on different cell types.

Identification of an inactive Δ CCR5 receptor

[0177] It is known that some individuals remain uninfected despite repeated exposure to HIV-1 [55, 56, 51]. A proportion of these exposed-uninfected individuals results from the relatively low risk of contamination after a single contact with the virus, but it has been postulated that truly resistant individuals do exist. In fact, CD4⁺ lymphocytes isolated from exposed-uninfected individuals are highly resistant to infection by primary M-tropic, but not T-tropic HIV-1 strains. Also, peripheral blood mononuclear cells (PBMC) from different donors are not infected equally with various HIV-1 strains [57-59]. Given the key role played by CCR5 in the fusion event that mediates infection by M-tropic viruses, it is postulated that variants of CCR5 could be responsible for the relative or absolute resistance to HIV-1 infection exhibited by some individuals, and possibly for the variability of disease progression in infected patients [66]. The Inventors selected three HIV-1 infected patients known to be slow progressors, and four seronegative individuals as controls; the full coding region of their CCR5 gene was amplified by PCR and sequenced. Unexpectedly, one of the slow progressors, but also two of the uninfected controls, exhibited heterozygosity at the CCR5 locus for a biallelic polymorphism. The frequent allele corresponded to the published CCR5 sequence, while the minor one displayed a 32 bp deletion within the coding sequence, in a region corresponding to the second extracellular loop of the receptor (Fig. 6). The figure 6 is the structure of the mutant form of human CC-chemokine receptor 5. *a*, The amino acid sequence of the non-functional Δ ccr5 protein is represented. The transmembrane organisation is given by analogy with the predicted transmembrane structure of the wild-type CCR5. Amino acids represented in black correspond to unnatural residues resulting from the frame shift caused by the deletion. The mutant protein lacks the last three transmembrane segments of CCR5, as well as the regions involved in G protein-coupling, *b*, Nucleotide sequence of the CCR5 gene surrounding the deleted region, and translation into the normal receptor (top) or the truncated mutant (*ccr5*, bottom). The 10-bp direct repeat is represented in italics. The full size coding region of the CCR5 gene was amplified by PCR, using 5'-TCGAGGATCCAAGATGGATTATCAAGT-3' and 5'-CTGATCTAGAGCCATGTGCACAACTCT-3' as forward and reverse primers respectively. The PCR products were sequenced on both strands using the same oligonucleotides as primers, as well as internal primers, and fluorochrome-labelled dideoxynucleotides as terminators. The sequencing products were run on an Applied Biosystem sequencer, and ambiguous positions were searched along the coding sequence. When the presence of a deletion was suspected from direct sequencing, the PCR products were cloned after restriction with *Bam*HI and *Xba*I endonucleases into pcDNA3. Several clones were sequenced to confirm the deletion. The deletion was identical in three unrelated individuals investigated by sequencing.

[0178] Cloning of the PCR product and sequencing of several clones confirmed the deletion. The deletion causes a frame shift, which is expected to result in premature termination of translation. The protein encoded by this mutant allele (Δ ccr5) therefore lacks the last three transmembrane segments of the receptor. A 10-bp direct repeat flanking the coding sequence for wild-type CCR5, the truncated *ccr5* mutant, the CCR2b or the Duffy chemokine receptors, or with the pcDNA3 vector alone. The target cells were also transfected with human CD4 expressed from the CMV promoter and the luciferase gene under the control of the T7 promoter. HeLa effector cells were infected (MOI = 10) with vaccinia vectors expressing T7-polymerase (vTF1.1) and either the JR-FL (vCB28) or 89.6 (vBD3) envelope proteins. The luciferase activity resulting from cell fusion is expressed as the percentage of the activity (in relative light units) obtained for wild-type CCR5. All transfections were performed with an identical quantity of plasmid DNA using pcDNA3 as carrier when necessary. To initiate fusion, target and effector cells were mixed in 24 well plates at 37 °C in the presence of ara-C and rifampicin, and allowed to fuse for 8 hours. Cells were lysed in 150 μ l of reporter lysis buffer (Promega) and assayed for luciferase activity according to the manufacturer's instructions (Promega).

[0179] Coexpression of Δ ccr5 with wild-type CCR5 consistently reduced the efficiency of fusion for both JR-FL and 89.6 envelopes, as compared with CCR5 alone. Whether this *in vitro* inhibitory effect (not shared by the chemokine receptor Duffy, used as control) also occurs *in vivo* is presently not known. Coexpression with the CCR2b receptor [31], which is the CC-chemokine receptor most closely related to CCR5 but does not promote fusion by M-tropic HIV-1 strains [48], did not rescue the mutation by formation of a hybrid molecule (Fig. 7).

[0180] The figure 8 represents genotyping of individuals by PCR and segregation of the CCR5 alleles in CEPH families. *a*, Autoradiography illustrating the pattern resulting from PCR amplification and *Eco*RI cleavage for individuals homozygous for the wild-type CCR5 allele (CCR5/CCR5), the null Δ ccr5 allele (Δ ccr5/ Δ ccr5), and for heterozygotes (CCR5/ Δ ccr5). A 735 bp PCR product is cleaved into a common band of 332 bp for both alleles, and into 403 and 371 bp bands for the wild-type and mutant alleles, respectively. *b*, Segregation of the CCR5 alleles in two informative families of the CEPH. Half-black and white symbols represent heterozygotes and wild-type homozygotes, respectively. For a few individuals in the pedigrees, DNA was not available (ND: not determined). PCRs were performed on genomic DNA samples, using 5'-CCTGGCTGTCGTCATGCTG-3' and 5'-CTGATCTAGAGCCATGTGCACAACCTCT-3' as forward and reverse primers respectively. Reaction mixtures consisted in 30 μ l of 10 mM Tris-HCl buffer pH 8.0, containing 50 mM KCl, 0.75 mM MgCl₂, 0.2 mM dCTP, dGTP and dTTP, 0.1 mM dATP, 0.5 μ i [α -³²P]-dATP, 0.01% gelatine, 5% DMSO, 200 ng target DNA, 60 ng of each of the primers and 1.5 U Taq polymerase. PCR conditions were: 93 °C for 2 min 30; 93 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min, 30 cycles; 72 °C for 6 min. After the PCR reaction, the samples were incubated for 60 min at 37 °C with 10 U *Eco*RI, and 2 μ l of the denatured reaction mixture was applied onto a denaturing 5% polyacrylamide gel containing 35% formamide and 5.6 M urea. Bands were detected by autoradiography.

[0181] Based on the 14 chromosomes tested in the first experiment, the deleted Δ ccr5 allele appeared rather frequent in the Caucasian population. The accurate frequency was further estimated by testing (Fig. 8a) a large cohort of Caucasian individuals, including unrelated members of the CEPH (Centre d'Etude des Polymorphismes Humains) families, part of the IRIBHN staff, and a bank of anonymous DNA samples from healthy individuals collected by the Genetics Department of the Erasme Hospital in Brussels. From a total of more than 700 healthy individuals, the allele frequencies were found to be 0.908 for the wild-type allele, and 0.092 for the mutant allele (Table I). The genotype frequencies observed in the population were not significantly different from the expected Hardy-Weinberg distribution (CCR5/CCR5: 0.827 vs 0.324; CCR5/ Δ ccr5: 0.162 vs 0.167; Δ ccr5/ Δ ccr5: 0.011 vs 0.008, $p > 0.999$), suggesting that the null allele has no drastic effect on fitness. Using two informative CEPH families, it was confirmed that the wild-type CCR5 gene and its Δ ccr5 variant were allelic, and segregated in a normal mendelian fashion (Fig. 8b). Interestingly, a cohort of 124 DNA samples originating from Central Africa (collected from Zaire, Burkina Fasso, Cameroun, Senegal and Benin) and Japan did not reveal a single Δ ccr5 mutant allele, suggesting that this allele is either absent or very rare in Asian, African black populations (Table I).

[0182] The consequences of the existence of a null allele of CCR5 in the normal Caucasian population were then considered in terms of susceptibility to infection by HIV-1. If, as it is predicted, CCR5 plays a major (not redundant) role in the entry of most primary virus strains into cells, then Δ ccr5/ Δ ccr5 individuals should be particularly resistant to HIV-1 challenge, both *in vitro* and *in vivo*. The frequency of the Δ ccr5/ Δ ccr5 genotype should therefore be significantly lower in HIV-1 infected patients, and increased in exposed-uninfected individuals. Also, if heterozygotes have a statistical advantage due to the lower number of functional receptors on their white blood cells, or to the possible dominant-negative properties of the mutant allele, the frequency of heterozygotes (and mutant alleles) should be decreased in HIV-infected populations. These hypotheses were tested by genotyping a large number of seropositive Caucasian individuals ($n = 645$) belonging to cohorts originating from various hospitals from Brussels, Liège and Paris (Table I). Indeed, it was found that within this large series, the frequency of the null Δ ccr5 allele was significantly reduced from 0.092 to 0.053 ($p < 10^{-5}$). The frequency of heterozygotes was also reduced from 0.162 to 0.106 ($p < 0.001$) and not a single Δ ccr5/ Δ ccr5 individual could be found ($p < 0.01$).

[0183] Altogether, functional and statistical data suggest that CCR5 is indeed the major co-receptor responsible for natural infection by M-tropic HIV-1 strains. Individuals homozygous for the null Δ ccr5 allele (about 1% of the Caucasian population) have apparently a strong resistance to infection. It is unclear at this point whether resistance to HIV-1 is absolute or relative, and whether resistance will vary depending on the mode of viral contamination. Larger cohorts of seropositive individuals will have to be tested in order to clarify this point. Heterozygotes have a milder though significant advantage: assuming an equal probability of contact with HIV, it can be inferred from Table I that heterozygotes have a 39% reduction in their likelihood of becoming seropositive, as compared to individuals homozygous for the wild-type CCR5 allele. Both a decrease in functional CCR5 receptor number, and a dominant-negative effect of Δ ccr5 *in vivo*, comparable to what is observed in the *in vitro* experiments (Fig. 7) are possible explanations for this relative protection. The mutant allele, which can be regarded as a natural knock-out in human, is not accompanied by an obvious phenotype in homozygous individuals. Nevertheless, the lack of overt phenotype, taken together with the relative protection that characterises heterozygous subjects, suggests that pharmacological agents that selectively block the ability of HIV-1 to utilise CCR5 as a cofactor, could be effective in preventing HIV-1 infection, and would be predicted not be associated with major side effects resulting from CCR5 inactivation. These pharmaceutical agents could be used with other compounds which are able to block other chemokine receptors used as co-receptors by some HIV-primary isolates in order to infect other cells [47]. The prevalence of the null allele in the Caucasian population raises the question of whether pandemics of HIV (or related viruses using the same co-receptor) have contributed during mankind's evolution to stabilise by selection the mutant ccr5 allele at such a high frequency.

Production of antibodies anti-CCR5

[0184] Antibodies were produced by genetic immunisation. Six week old females balb/c mice were used. DNA coding for the human CCR5 receptor was inserted in the expression vector pcDNA3 under the control of the CMV promotor and 100 µg DNA was injected in the anterior tibial muscle, five days after pre-treatment of this muscle with cardiotoxine (from venom of *Naja Nigricolis*). Injections were repeated twice at three week intervals. Fifteen days after the last injection, blood was taken from each animal and sera were tested for the presence of anti-CCR5 antibodies.

Test of sera using Fluorescence Activated Cell Sorter (FACS)

[0185] Sera were tested by fluorescence activated cell sorting using recombinant CHO cells expressing the CCR5 receptor. Briefly, cells were detached using a PBS-EDTA-EGTA solution and incubated into PBS-BSA medium for 30 minutes at room temperature with 5 µl serum on the basis of 100,000 cells per tube. Cells were then washed and incubated for 30 minutes in ice together with anti-mouse antibody labelled with fluorescein. Cells were washed, taken up into 200 µl of a PBS-BSA solution and fluorescence was analysed by FACS (FACSCAN, Becton-Dickinson). 10,000 cells were counted. Wild type CHO or recombinant CHO cells expressing the human CCR2b receptor were used as controls.

[0186] When tested by FACS analysis 2 weeks after the last injection (figure 9), all the sera from mice immunised with CCR5 cDNA, clearly recognised the native receptor expressed on CHO cells (mean of fluorescence = 200), without significant cross reaction with control cells expressing CCR2b (mean of fluorescence = 20).

[0187] Sera were tested on either a CHO cell line expressing high level of CCR5 receptor (black histogram) or a CHO cell line expressing CCR2b receptor (white histogram) as negative control. Each serum was tested individually.

Antibodies anti-CCR5 and HIV infectivity

[0188] Peripheral blood mononuclear cells (PBMC) from one donor homozygous from wild type CCR5 gene, were isolated and cultivated 3 days in presence of PHA.

[0189] On day 4, 800 µl of cells (10^5 cells/ml) were incubated with 8 µl of sera from mice immunised with CCR5 cDNA, 30 minutes at 37 °C. 1 ml of viral solution (JRCSF HIV strain) is then added and incubated during 2 hours. Cells were then washed twice and cultivated during 15 days.

[0190] Aliquot of medium is taken at days 0, 4, 7, 10 and 14 and the dosage of antigen p24 is performed.

[0191] 14 days after the beginning of the experiment, one serum (serum B0) totally block the production of p24, indicating its ability to block the infection of the lymphocytes by this HIV strain (figure 10). Other serums also exhibit a partial or total effect on this infection (serum A2 and B1). All the other sera did not show any effect on this infection.

Production of monoclonal antibodies

[0192] Mice with the highest titre of CCR5 antibodies were selected for monoclonal antibodies production and injected intravenously with 10^7 recombinant CHO-K1 cells expressing human CCR5 receptors. Three days later, animals were sacrificed and fusion of splenic cells or cells from lymph nodes near the site of injection with SP2/0 myeloma cells, were performed. Fusion protocol used was that of Galfre et al. (Nature 266, 550 (1977)). A selective HAT (hypoxanthine/aminopterin/thymidin) medium is used to select hybridomas and their supernatants are tested by FACS using recombinant CHO cells expressing the human CCR5 receptor, as it was done for the sera. Positives hybridomas are then cloned by limited dilution. Clones that are shown positive by FACS analyses are then expanded and produced in ascites in balb/C mice.

Table 1

	Seronegative			Seropositive			Chi-squared
	Number	Frequency	Standard error	Number	Frequency	Standard error	
Genotypes :							
CCR5/CCR5	582	0.827	0.014	645	0.892	0.012	2 degrees of freedom 17.7 p < 0.0005
CCR5/Δ CCR5	114	0.162	0.014	78	0.108	0.012	
CCR5/Δ CCR5	8	0.011	0.004	0	0.000	< 0.001	
Total :	704	1.000		723	1.000		
Alleles :							
CCR5	1278	0.908	0.008	1368	0.946	0.006	1 degree of freedom p < 0.0005
Δ CCR5	130	0.092	0.008	78	0.054	0.006	
Total :	1408	1.000		1446	1.000		

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Claims

1. A method for determining whether an anti-ligand is capable of inhibiting the binding of a ligand, said ligand being an HIV virus or a portion thereof, to a peptide having an amino acid sequence which presents more than 80% homology with SEQ ID NO. 2 shown in figure 1, said method comprising the steps of contacting a cell transfected with a vector expressing the nucleic acid molecule encoding said peptide, with the anti-ligand under conditions permitting a binding of the anti-ligand to said peptide and determining whether the said anti-ligand inhibits the binding of the said ligand, to said peptide.
2. A method for determining whether an anti-ligand is capable of inhibiting the binding of a ligand, said ligand being an HIV virus or a portion thereof, to a peptide having an amino acid sequence which presents more than 80% homology with SEQ ID NO. 2 shown in figure 1, said method comprising the steps of:

 - preparing a cell extract from cells transfected with a vector expressing the nucleic acid molecule encoding said peptide,

- isolating a membrane fraction from the cell extract,
 - contacting the anti-ligand with the membrane fraction under conditions permitting binding of the anti-ligand to said peptide, and
 - determining whether the said anti-ligand inhibits the binding of the said ligand to said peptide.
- 5
3. Method according to claim 1 or 2, wherein the method comprises the steps of:
- preparing a cell or cell extract, said cell being transfected with the nucleic acid molecule encoding a peptide having an amino acid sequence which presents more than 80% homology with SEQ ID NO. 2 shown in figure 1,
 - 10 - possibly isolating a membrane fraction from the cell extract,
 - contacting the cell or membrane fraction with said anti-ligand, in the presence of the said ligand of said peptide, under conditions permitting the activation of a functional peptide response, and
 - detecting by means of a bio-assay a modification in the activity of the peptide, thereby determining whether the said anti-ligand inhibits the binding of the said ligand to said peptide.
- 15
4. A method of screening drugs to identify drugs which specifically bind the peptide comprising the amino acid sequence which presents more than 80% homology with SEQ ID NO. 2 shown in figure 1, or a part thereof, and can be used in the treatment and/or prevention of HIV virus infection, said method comprising contacting a cell transfected with a vector expressing the nucleic acid molecule encoding said peptide, with a drug under conditions permitting binding of said drug to said peptide and determining whether said drug specifically binds the transfected cell, thereby identifying a drug which specifically binds to said peptide and which can be used in the treatment and/or prevention of HIV virus infection.
- 20
5. A method of screening drugs to identify drugs which specifically bind the peptide comprising the amino acid sequence which presents more than 80% homology with SEQ ID NO. 2 shown in figure 1, or a part thereof, and can be used in the treatment and/or prevention of HIV virus infection, said method comprising preparing a cell extract from cells transfected with a vector expressing the nucleic acid molecule encoding said peptide, isolating a membrane fraction from the cell extract, contacting the membrane fraction with a drug under conditions permitting binding of said drug to said peptide and determining whether said drug specifically binds the transfected cell, thereby identifying a drug which specifically binds to said peptide and which can be used in the treatment and/or prevention of HIV virus infection.
- 25
6. A method for determining whether an agonist or antagonist which binds to a peptide comprising the amino acid sequence which presents more than 80% homology with SEQ ID NO. 2 shown in figure 1, or a part thereof, can be used for the treatment and/or prevention of an HIV virus infection, said method comprising contacting a cell transfected with a vector expressing the nucleic acid molecule encoding said peptide with the agonist or antagonist under conditions permitting binding of the agonist or antagonist to said peptide and determining whether the said agonist or antagonist inhibits the binding of HIV virus to said peptide.
- 30
7. A method for determining whether an agonist or antagonist which binds to a peptide comprising the amino acid sequence which presents more than 80% homology with SEQ ID NO. 2 shown in figure 1, or a part thereof, can be used for the treatment and/or prevention of an HIV virus infection, said method comprising preparing a cell extract from cells transfected with a vector expressing the nucleic acid molecule encoding said peptide, isolating a membrane fraction from the cell extract, contacting the membrane fraction with the agonist or antagonist under conditions permitting binding of the agonist or antagonist to said peptide and determining whether the said agonist or antagonist inhibits the binding of HIV virus to said peptide.
- 35
8. The method according to any of the claims 1 to 7, wherein said HIV virus is selected from the group consisting of the human immunodeficiency virus 1 (HIV 1), the human immunodeficiency virus 2 (HIV 2) or a portion of said HIV viruses.
- 40
9. The method according to any of the claims 1 to 8, wherein the portion of said HIV virus is the glycoprotein GP120/GP160 or a portion thereof.
- 45
10. The method according to any of the claims 1 to 9, further comprising the step of measuring the infectivity of the cell by an HIV strain wherein said anti-ligand, drug, agonist or antagonist decreases infectivity by said HIV strain.
- 50
11. The method according to claim 10, wherein the cell is a lymphocyte cell.
- 55

12. The method according to claim 10 or 11, wherein the HIV strain is the human immunodeficiency virus 1 (HIV-1).
13. The method according to claim 10 or 11, wherein the HIV strain is the human immunodeficiency virus 2 (HIV-2).
- 5 14. The method according to any of the claims 10 to 13, wherein the decrease in HIV infectivity is measured by the dosage of an HIV protein.
15. The method according to claim 14, wherein said HIV protein is the HIV antigen P24.
- 10 16. The method according to any of the claims 1 to 15, wherein the amino acid sequence of the peptide presents more than 90% homology with SEQ ID NO. 2 shown in figure 1.
17. The method according to any of the claims 1 to 15, wherein the amino acid sequence of the peptide presents more than 95% homology with SEQ ID NO. 2 shown in figure 1.
- 15 18. The method according to any of the claims 1 to 15, wherein the amino acid sequence of the peptide is the amino acid sequence SEQ ID NO. 2 shown in figure 1 or a portion thereof comprising at least the N-terminus segment and the first extracellular loop of the amino acid sequence SEQ ID NO. 2 shown in figure 1.
- 20 19. The method according to claim 3, wherein the modification in the peptide activity is detected by a bio-assay based upon the modification in a production of a second messenger.
20. The method of claim 19, wherein the bio-assay is based upon a measurement of calcium ions or inositol phosphates (such as IP₃) concentration.
- 25 21. The method according to any one of the claims 1 to 20, wherein the cell is a mammalian cell non neuronal in origin, preferably selected from the group consisting of CHO-K1, HEK293, BHK21 and COS-7 cells.
22. The method according to any of the claims 1 to 21, wherein the anti-ligand, the drug, the agonist or the antagonist is an antibody.
- 30 23. The method according to claim 22, wherein the antibody is a monoclonal antibody.
24. The method according to claim 23, wherein the monoclonal antibody is directed to an epitope of the said peptide present on the surface of a cell.
- 35 25. An antibody which inhibits or reduces the binding of the human immunodeficiency virus 1 (HIV 1) or the human immunodeficiency 2 (HIV 2) to a peptide having an amino acid sequence presenting more than 80% homology with SEQ ID NO. 2 shown in figure 1, identified according to the method of any of the claims 1 to 24.
- 40 26. The antibody according to the claim 25, wherein the amino acid sequence of the peptide presents more than 90% homology with SEQ ID NO. 2 shown in figure 1.
27. The antibody according to the claim 25, wherein the amino acid sequence of the peptide presents more than 95% homology with SEQ ID NO. 2 shown in figure 1.
- 45 28. The antibody according to the claim 25, wherein the amino acid sequence of the peptide is the amino acid sequence SEQ ID NO. 2 shown in figure 1.
- 50 29. The antibody according to any of the claims 25 to 28, which is a monoclonal antibody.
30. The antibody according to claim 29, which is a monoclonal antibody directed to an epitope of the said peptide, present on the surface of a cell expressing said peptide.
- 55 31. The antibody according to any of the claims 25 to 30, which decreases infectivity of a cell by an HIV strain.
32. The antibody according to claim 31, wherein the cell is a lymphocyte cell.

33. The antibody according to claim 31 or 32, wherein the HIV strain is a human immunodeficiency virus 1 (HIV-1 strain).
34. The antibody according to claim 31 or 32, wherein the HIV strain is a human immunodeficiency virus 2 (HIV-2 strain).
- 5 35. A pharmaceutical composition comprising an adequate pharmaceutical carrier and a sufficient amount of the antibody according to any of the claims 25 to 34.
36. The use of the pharmaceutical composition according to the claim 35 for the manufacture of a medicament in the prevention and/or the treatment of an infection by the human immunodeficiency virus 1 (HIV-1) and/or the human
10 immunodeficiency virus 2 (HIV-2) (AIDS).
37. Use of an antisense oligonucleotide, comprising a sequence which specifically hybridizes to a nucleic acid molecule having more than 80% homology with nucleic acid sequence SEQ ID NO. 2 shown in figure 1, for the manufacture of a medicament in the prevention and/or the treatment of an infection by the human immunodeficiency virus 1
15 (HIV-1) and/or the human immunodeficiency virus 2 (HIV-2) (AIDS).
38. Use of an antisense oligonucleotide, comprising a sequence which specifically hybridizes to a nucleic acid molecule having at least the nucleic acid sequence SEQ ID NO. 2 shown in figure 1 or a portion thereof, for the manufacture of a medicament in the prevention and/or the treatment of an infection by the human immunodeficiency virus 1
20 (HIV-1) and/or the human immunodeficiency virus 2 (HIV-2) (AIDS).
39. The use according to the claim 37 or 38, wherein said nucleic acid molecule encodes a peptide as defined in any of the claims 1 to 7 and 16 to 18.
- 25 40. The use according to any of the claims 37 to 39, wherein said nucleic acid molecule is a cDNA molecule or a genomic DNA molecule.
41. The use according to any of the claims 37 to 40, wherein said antisense oligonucleotide comprises chemical
30 analogs of nucleotides.

SEQ ID NO.1

FIG.1 a

GAATTC	CCCCCAACAGAGCCAAGCTCTCCATCTAGTGGACAGGGAAGCTAGCAGCAAACC	59
		19
TTCCCTTCACTACAAA	ACTTCATTGCTTGGCCAAAAAGAGAGTTAATTCAATGTAGACAT	119
		39
CTATGTAGGCAATTAAAA	ACCTATTGATGTATAAAACAGTTTGCAATCATGGAGGGCAAC	179
		59
TAAATACATTCTAGGACTTTATA	AAAAGATCACTTTTTATTATGACAGGGTGGAAACAAG	239
		79
ATGGATTATCAAGTGTCAAGTCCAATCTATGACATCAATTATTATACATCGGAGCCCTGC		299
M D Y Q V S S P I Y D I N Y Y T S S P C		99
CAAAAAATCAATGTGAAGCAAATCGCAGCCCGCCTCCTGCCTCCGCTCTACTCACTGGTG		359
Q K I N V K Q I A A R L L P P L Y S L V		119
TTCATCTTTGGTTTTGTGGGCAACATGCTGGTCACTCCTCATCCTGATAAACTGCAAAAGG		419
F I F G F V G N M L V I L I L I N C K R		139
CTGAAGAGCATGACTGACATCTACCTGCTCAACCTGGCCATCTCTGACCTGTTTTTCCTT		479
L K S M T D I Y L L N L A I S D L F F L		159
CTTACTGTCCCCTTCTGGGCTCACTATGCTGCCGCCAGTGGGACTTTGGAAATACAATG		539
L T V P F W A H Y A A A Q W D F G N T M		179
TGTCAACTCTTGACAGGGCTCTATTTTATAGGCTTCTTCTCTGGAATCTTCTTCATCATC		599
C Q L L T G L Y F I G F F S G I F F I I		199
CTCCTGACAATCGATAGGTACCTGGCTGTGCTCCATGCTGTGTTTGCTTTAAAAGCCAGG		659
L L T I D R Y L A V V H A V F A L K A R		219
ACGGTCACCTTTGGGGTGGTGACAAGTGTGATCACTTGGGTGGTGGCTGTGTTTGCGTCT		719
T V T F G V V T S V I T W V V A V F A S		239
CTCCCAGGAATCATCTTTACCAGATCTCAAAAAGAAGTCTTCATTACACCTGCAGCTCT		779
L P G I I F T R S Q K E G L H Y T C S S		259
CATTTTCCATACA		
H F P Y		

GAATTCCTCCCAACAGAGCCAAGCTCTCCATCTAGTGGACAGGGAAGCTAGCAGCAAACC	59
	19
TTCCCTTCACTACAAAACCTTCATTGCTTGGCCAAAAAGAGAGTTAATTCAATGTAGACAT	119
	39
CTATGTAGGCAATTAAAAACCTATTGATGTATAAAACAGTTTGCATTTCATGGAGGGCAAC	179
	59
TAAATACATTCTAGGACTTTATAAAAGATCACTTTTTTATTTATGCACAGGGTGAACAAG	239
	79
ATGGATTATCAAGTGTCAAGTCCAATCTATGACATCAATTATTATACATCGGAGCCCTGC	299
M D Y Q V S S P I Y D I N Y Y T S E P C	99
CAAAAAATCAATGTGAAGCAAATCGCAGCCCGCCTCCTGCCTCCGCTCTACTCACTGGTG	359
Q K I N V K Q I A A R L L P P L Y S L V	119
TTCACTCTTTGGTTTTGTGGGCAACATGCTGGTCATCCTCATCCTGATAAACTGCAAAAGG	419
F I F G F V G N M L V I L I L I N C K R	139
CTGAAGAGCATGACTGACATCTACCTGCTCAACCTGGCCATCTCTGACCTGTTTTTCCTT	479
L K S M T D I Y L L N L A I S D L F F L	159
CTTACTGTCCCCCTTCTGGGCTCACTATGCTGCCGCCAGTGGGACTTTGGAAATACAATG	539
L T V P F W A H Y A A A Q W D F G N T M	179
TGTCAACTCTTGACAGGGCTCTATTTTATAGGCTTCTTCTCTGGAATCTTCTTCATCATC	599
C Q L L T G L Y F I G P F S G I P F I I	199
CTCCTGACAAATCGATAGGTACCTGGCTGTCGTCCATGCTGTGTTTGCTTTAAAAGCCAGG	659
L L T I D R Y L A V V H A V F A L K A R	219
ACGGTCACCTTTGGGGTGGTGACAAGTGTGATCACTTGGGTGGTGGCTGTGTTTGGCTCT	719
T V T F G V V T S V I T W V V A V F A S	239
CTCCCAGGAATCATCTTTACCAGATCTCAAAAAGAAGGTCTTCATTACACCTGCAGCTCT	779
L P G I I F T R S Q K E G L H Y T C S S	259
CATTTTCCATACAGTCAGTATCAATTCTGGAAGAATTTCCAGACATTAAAGATAGTCATC	839
H F P Y S Q Y Q F W K N F Q T L K I V I	279

SEQ ID NO.2 FIG.1b

TTGGGGCTGGTCCTGCCGCTGCTTGTTCATGGTCATCTGCTACTCGGGAATCCTAAAACT	899
L G L V L P L L V M V I C Y S G I L K T	299
CTGCTTCGGTGTTCGAAATGAGAAGAAGAGGCACAGGGCTGTGAGGCTTATCTTCACCATC	959
L L R C R N E K K R H R A V R L I F T I	319
ATGATTGTTTATTTTCTCTTCTGGGCTCCCTACAACATTGTCCTTCTCCTGAACACCTTC	1019
M I V Y F L F W A P Y N I V L L L N T F	339
CAGGAATTCTTTGGCCTGAATAATTGCAGTAGCTCTAACAGGTTGGACCAAGCTATGCAG	1079
Q E F F G L N N C S S S N R L D Q A M Q	359
GTGACAGAGACTCTTGGGATGACGCACTGCTGCATCAACCCCATCATCTATGCCTTTGTC	1139
V T E T L G M T H C C I N P I I Y A F V	379
GGGGAGAAGTTCAGAACTACCTCTTAGTCTTCTTCCAAAAGCACATTGCCAAACGCTTC	1199
G E K F R N Y L L V F F Q K H I A K R F	399
TGCAAATGCTGTTCATTTTTCCAGCAAGAGGCTCCCGAGCGAGCAAGCTCAGTTACACC	1259
C K C C S I F Q Q E A P E R A S S V Y T	419
CGATCCACTGGGGAGCAGGAAATATCTGTGGGCTTGTGACACGGACTCAAGTGGGCTGGT	1319
R S T G E Q E I S V G L *	439
GACCCAGTCAGAGTTGTGCACATGGCTTAGTTTTATACACAGCCTGGGCTGGGGGTNGG	1379
	459
TTGGMNGAGGTCTTTTTTAAAAGGAAGTTACTGTTATAGAGGGTCTAAGATTCATCCATT	1439
	479
TATTTGGCATCTGTTTAAAGTAGATTAGATCCGAATTC	

SEQ ID NO.2 (SUITE)

FIG.1c

GAATTCCCCAACAGAGCCAAGCTCTCCATCTAGTGGACAGGGAAGCTAGCAGCAAACC	59
	19
TTCCCTTCACTACAAAACCTTCATTGCTTGGCCAAAAAGAGAGTTAATTCAATGTAGACAT	119
	39
CTATGTAGGCAATTAAAAACCTATTGATGTATAAACAGTTTGCAATTCATGGAGGGCAAC	179
	59
TAAATACATTCTAGGACTTTATAAAAGATCACTTTTATTTATGCACAGGGTGGAAACAAG	239
	79
ATGGATTATCAAGTGTCAAGTCCAATCTATGACATCAATTATTATACATCGGAGCCCTGC	299
M D Y Q V S S P I Y D I N Y Y T S E P C	99
CAAAAAATCAATGTGAAGCAAATCGCAGCCCGCCTCCTGCTCCGCTCTACTCACTGGTG	359
Q K I N V K Q I A A R L L P P L Y S L V	119
TTCACTCTTGGTTTTGTGGGCAACATGCTGGTCATCCTCATCTGATAAACTGCAAAGG	419
F I F G F V G N M L V I L I L I N C K R	139
CTGAAGAGCATGACTGACATCTACCTGCTCAACCTGGCCATCTCTGACCTGTTTTCTCTT	479
L K S M T D I Y L L N L A I S D L F F L	159
CTTACTGTCCCCTTCTGGGCTCACTATGCTGCCGCCAGTGGGACTTTGGAAATACAATG	539
L T V P F W A H Y A A A Q W D F G N T M	179
TGTCAACTCTTGACAGGGCTCTATTTTATAGGCTTCTCTCTGGAATCTTCTTCATCATC	599
C Q L L T G L Y F I G F F S G I F F I I	199
CTCCTGACAATCGATAGGTACCTGGCTGCTGCTCCATGCTGTGTTTGCTTTAAAGCCAGG	659
L L T I D R Y L A V V H A V P A L K A R	219
ACGGTCACCTTTGGGGTGGTGACAAGTGTGATCACTTGGGTGGTGGCTGTGTTTGGCTCT	719
T V T F G V V T S V I T W V V A V P A S	239
CTCCAGGAATCATCTTTACCAGATCTCAAAAAGAAGGTCTTCATTACACCTGCAGCTCT	779
L P G I I F T R S Q K E G L H Y T C S S	259
CATTTTCCATACATTAAAGATAGTCATCTTGGGGCTGGTCTGCGCTGCTTGTTCATGGT	839
H F P Y I K D S H L G A G P A A A C H G	279

SEQ ID NO.3

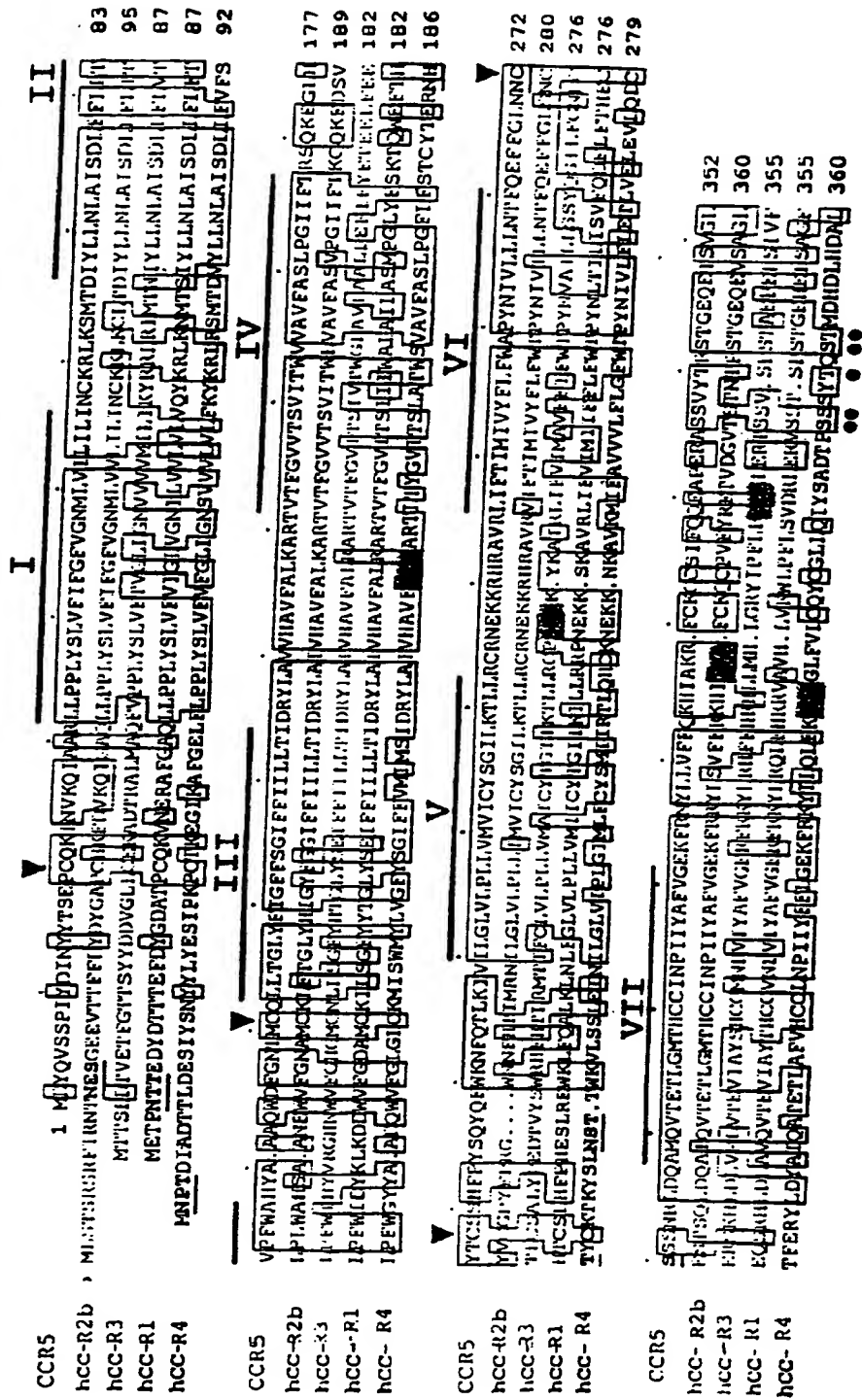
FIG.1d

CATCTGCTACTCGGGAATCCTAAAACTCTGCTTCGGTGTGCGAAATGAGAACTAGAGGGCA	899
H L L L G N P K N S A S V S K *	299
CAGGGCTGTGAGGCTTATCTTCACCATCATGATTGTTTATTTCTCTTCTGGGCTCCCTA	959
	319
CAACATTGTCTTCTCCTGAACACCTTCAGGAATTCTTTGGCCTGAATAATTGCAGTAG	1019
	339
CTCTAACAGGTTGGACCAAGCTATGCAGGTGACAGAGACTCTTGGGATGACGCACTGCTG	1079
	359
CATCAACCCCATCATCTATGCCTTTGTGCGGGGAGAAGTTCAGAACTACCTCTTAGTCTT	1139
	379
CTTCCAAAAGCACATTGCCAAACGCTTCTGCAATGCTGTTCTATTTCCAGCAAGAGGC	1199
	399
TCCCGAGCGAGCAAGCTCAGTTTACACCCGATCCACTGGGGAGCAGGAAATATCTGTGGG	1259
	419
CTTGTGACACGGACTCAAGTGGGCTGGTGACCCAGTCAGAGTTGTGCACATGGCTTAGTT	1319
	439
TTTATACACAGCCTGGGCTGGGGGTNGGTTGGNNGAGGTCTTTTAAAGGAAGTTACT	1379
	459
GTTATAGAGGGTCTAAGATTCATCCATTTATTGGCATCTGTTTAAAGTAGATTAGATCC	1439
	479
GAATTC	

SEQ ID NO.3 (SUITE)

FIG.1e

FIG. 2



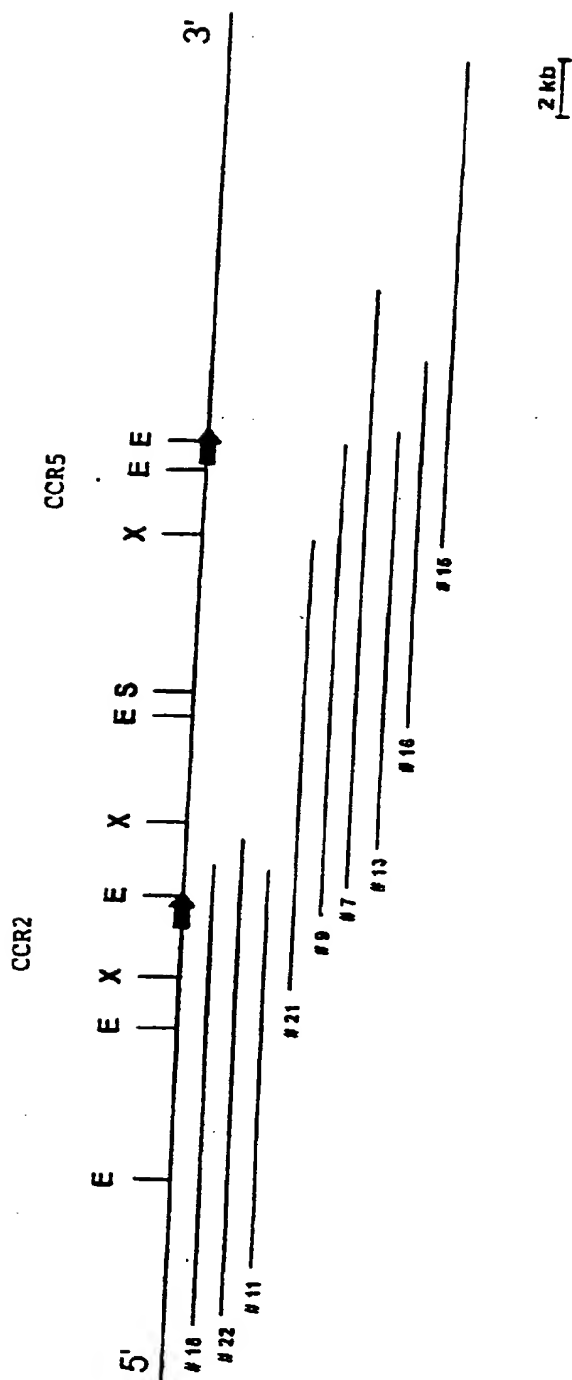


FIG. 3

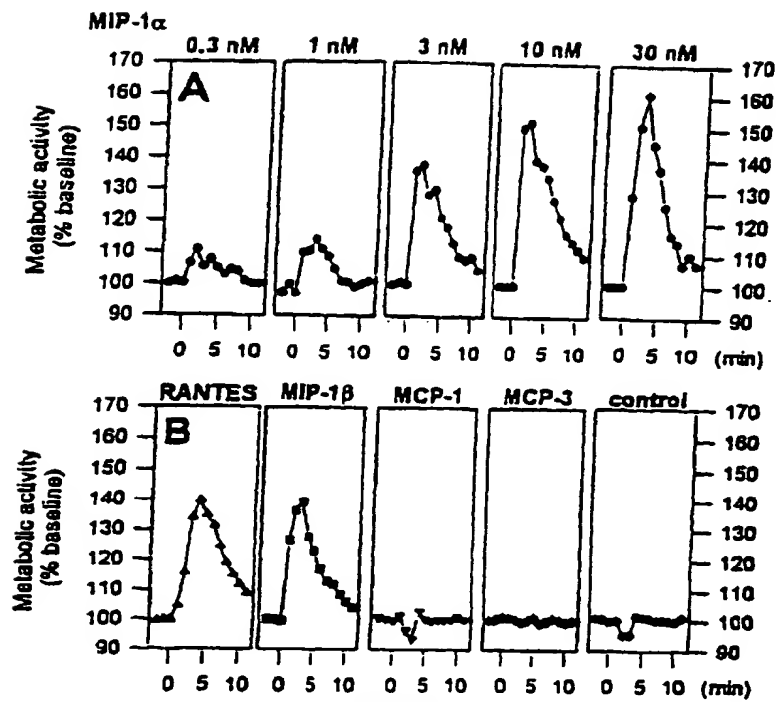


FIG. 4a

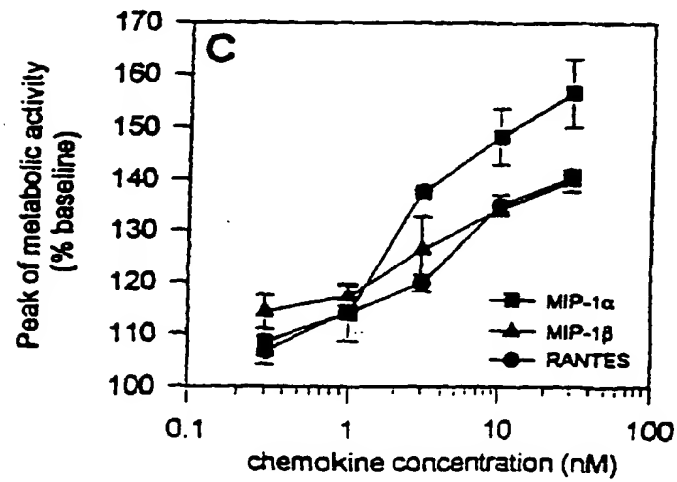


FIG. 4b

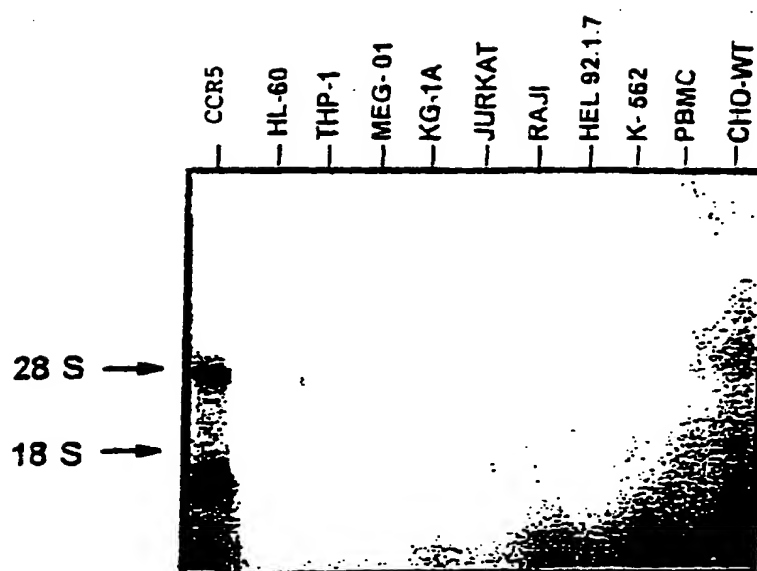


FIG. 5

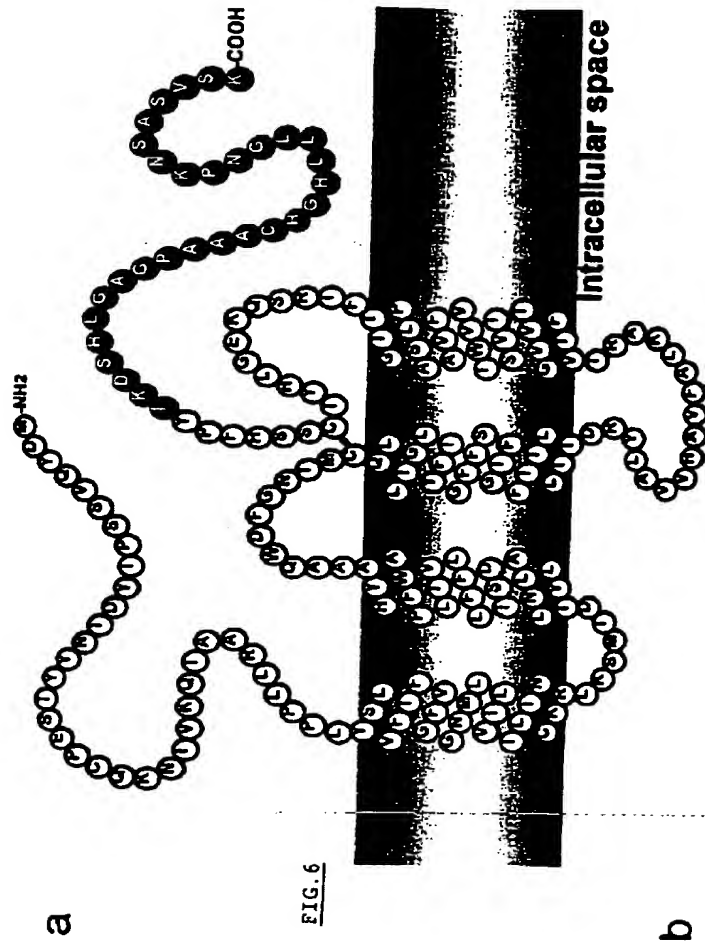
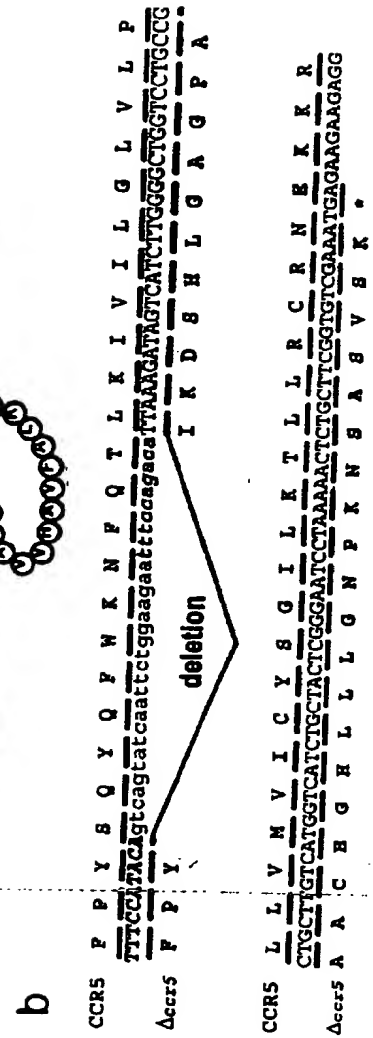


FIG. 6



A.

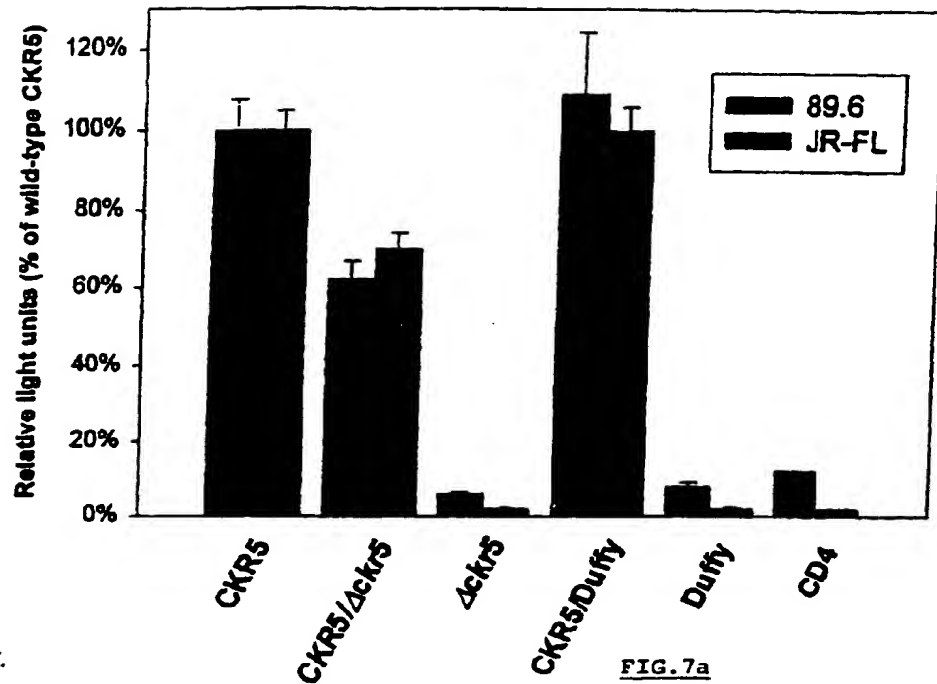


FIG. 7a

B.

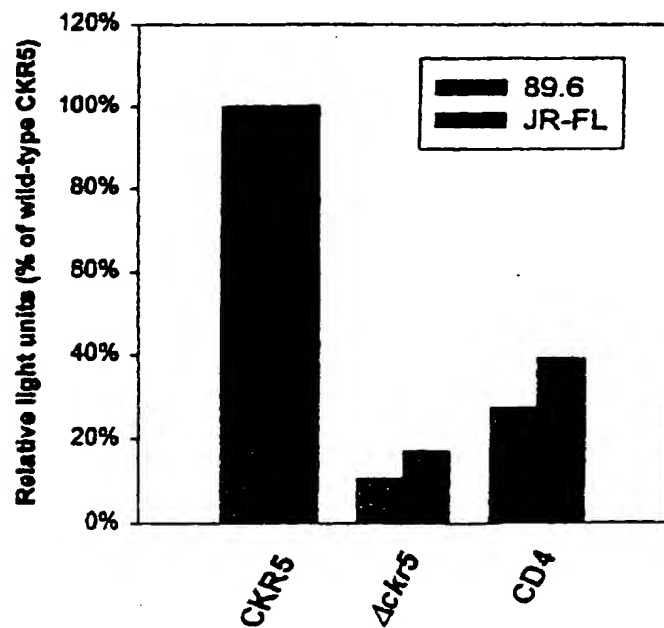


FIG. 7b

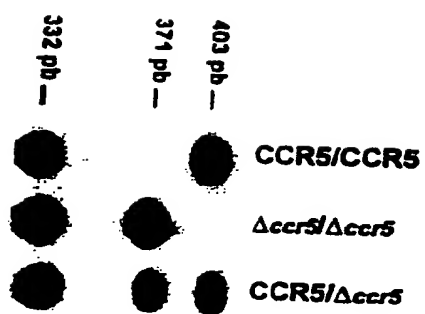


FIG. 8

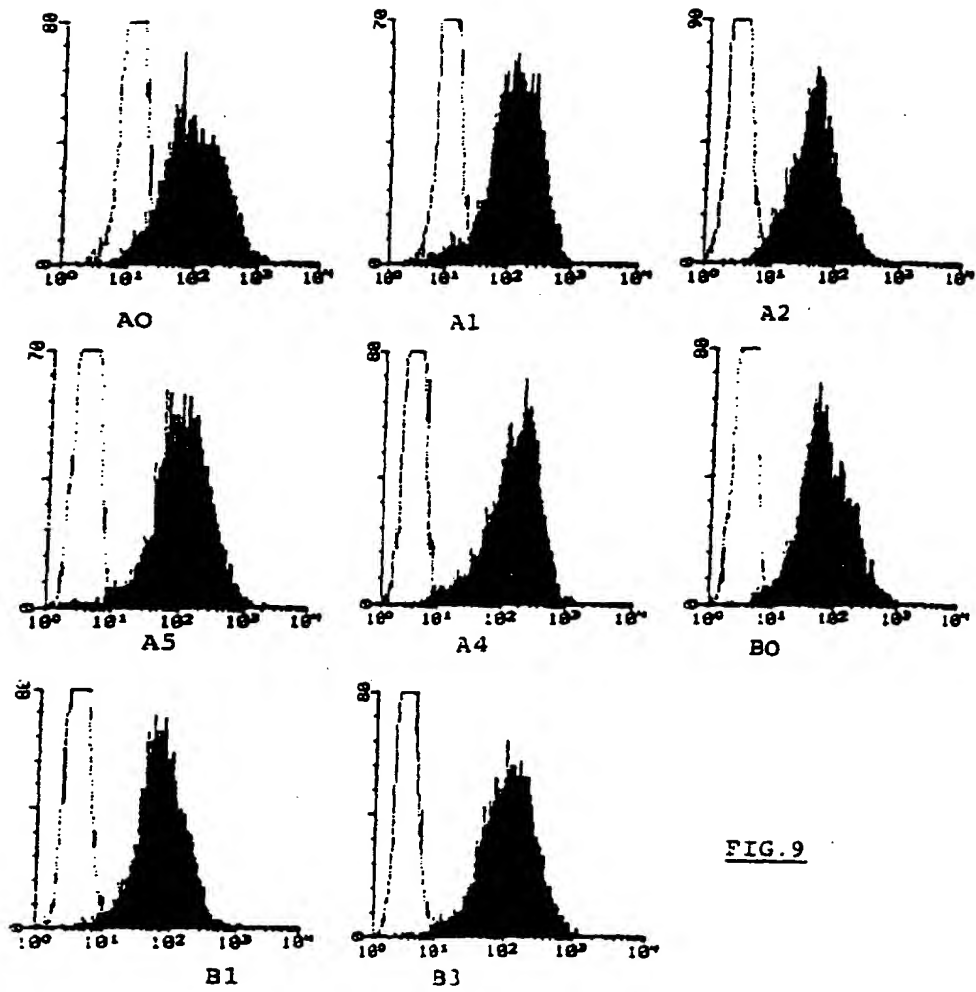
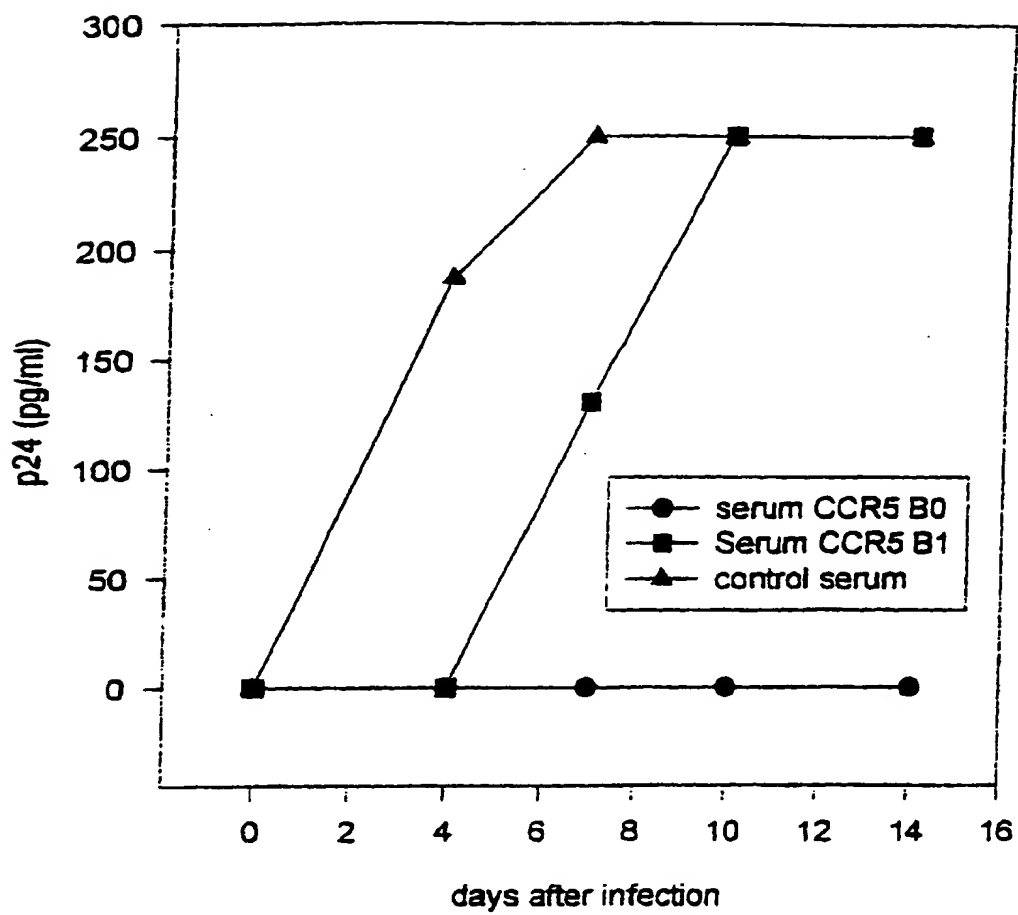


FIG. 9

FIG.10



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The present search report has been drawn up for all claims			
Place of search The Hague		Date of completion of the search 8 October 2004	Examiner Holtorf, S
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The present search report has been drawn up for all claims			
Place of search		Date of completion of the search	Examiner
The Hague		8 October 2004	Holtorf, S
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone</p> <p>Y : particularly relevant if combined with another document of the same category</p> <p>A : technological background</p> <p>O : non-written disclosure</p> <p>P : intermediate document</p> <p>T : theory or principle underlying the invention</p> <p>E : earlier patent document, but published on, or after the filing date</p> <p>D : document cited in the application</p> <p>L : document cited for other reasons</p> <p>& : member of the same patent family, corresponding document</p>			

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E	WO 97/45543 A (COMBADIÈRE CHRISTOPHE ; FENG YU (US); US HEALTH (US); ALKHATIB GHAL) 4 December 1997 (1997-12-04) page 26, 44, examples * page 4 * * page 21 - page 25 * * page 28 - page 31 * * example 3 * * example 4 * * example 5 *	1-8, 10, 12, 13, 16-35	
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The present search report has been drawn up for all claims			
Place of search The Hague		Date of completion of the search 8 October 2004	Examiner Holtorf, S
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